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Neural Mechanism of Play Fighting – Neural Circuitry, Vasopressin, and CRH – in Juvenile Golden Hamsters

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Neural Mechanism of Play Fighting – Neural Circuitry, Vasopressin, and CRH – in Juvenile Golden Hamsters

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Play fighting is common in juvenile mammals as a peri-pubertal form of agonistic behavior preceding adult aggressive behavior. In golden hamsters, play fighting peaks in early puberty around postnatal day 35 (P-35), and gradually matures into adult aggression in late puberty. Though extensively studied, the neural mechanisms underlying play fighting remains poorly understood. My dissertation focuses on identifying the neural circuitry and neural transmitter systems that mediate this behavior in juvenile golden hamsters.

Based on behavioral similarities between the offensive components of play fighting and adult aggression, I predicted that the neural circuitries mediating both behaviors shared common components. This possibility was tested by quantifying the immunolabeling of *c*-Fos expression in juvenile hamsters after the consummation of play fighting. In support of my hypothesis, I found that areas previously associated with

offensive aggression in adult hamsters, including the ventrolateral hypothalamus (VLH), the posterior dorsal part of the medial amygdala (MePD), and the bed nucleus of the stria terminalis (BST), also showed enhanced *c*-Fos expression after play fighting, which supported my hypothesis.

Vasopressin (AVP) facilitates aggression in adult hamsters. Therefore, I hypothesized that AVP also activates play fighting. To test my hypothesis, juvenile male golden hamsters were tested for play fighting after they received central microinjections of an AVP V1A-receptor antagonist into the anterior hypothalamus (AH). Also, immunocytochemistry was performed to identify possible AVP neurons associated with this behavior. I found that the AVP antagonist selectively inhibited the attack components of play fighting in experimental animals. In addition, AVP cells in the nucleus circularis (NC) and the medial division of the supraoptic nucleus (mSON), which were associated with offensive aggression, also showed increased c-Fos activity after play fighting. Together, these results show that AVP facilitates offensive behaviors throughout hamster development, from play fighting in juveniles to aggression in adults.

A recent study shows that oral administration of a CRH receptor antagonist inhibits aggression in adult hamsters. Therefore, I predicted that CRH plays a similar role in play fighting. To test my prediction, juvenile hamsters were tested for play fighting after central microinjections of a CRH receptor antagonist. I found that microinjections of the CRH receptor antagonist within the lateral septum (LS) resulted in an inhibition of several aspects of play fighting. The possible source of CRH affecting the behavior was tested through combined immunocytochemistry to CRH and c-Fos. I

found CRH neurons in the diagonal band of Broca (DBB), an area with extensive connections with the LS, were particularly activated in association with play fighting.

In conclusion, I find that shared neural elements participating in the "vertebrate social behavior neural network" are associated with both aggression and play fighting in hamsters. This circuitry is activated before the onset of puberty and is affected by rising levels of steroid hormones during the developmental period leading to adult behaviors. Within the circuitry, vasopressin release in the AH appears to control the activation of play fighting attacks. In contrast, CRH release in the LS affects a broader range of aspects of play fighting, including not just consummatory aspects of the behavior, but apparently also appetitive components in the form of contact duration.

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Chapter 1: Introduction

Play Fighting

Play behaviors are very common among mammals, and are classified under three different categories: object play, locomotor play, and play fighting. Play fighting, a form of agonistic behavior, refers to play directly with conspecifics (Fagen, 1981; Vanderschuren et al., 1997). This behavior is one of the earliest forms of non-mother-directed social behavior observed in mammals (Pellis and Pellis, 1991; Vanderschuren et al., 1997). One of the characteristics of play fighting is its reward value. Play fighting has been used as a reinforcer for rats in maze learning and place-preference conditioning (Calcagnetti and Schechter, 1992; Humphreys and Einon, 1981; Normansell and Panksepp, 1990). Behaviorally, play fighting includes the same offensive and defensive components as adult aggression (Pellis, 2002; Delville et al., 2005). The complexity of play fighting differs greatly between species. In rats, play fighting includes sophisticated attack and defense repertoires between players (Pellis et al., 1989). In mice, play fighting is limited to only chasing and evasion (Pellis and Pasztor, 1999).

Play Fighting in Rats

In rats, the goal of the offensive component of play fighting consists primarily of nuzzling the nape of the neck and the defensive component consists of preventing

the nape from being contacted (Pellis and Pellis, 1987). Developmentally, this behavior begins before weaning at around P-18 (Bolls and Wools, 1964; Pellis and Pellis, 1997), peaks between P-30 to P-40, and wanes at puberty at around P-50 to P-60 (Panksepp, 1981). The development can be divided into three separated phases: an undifferentiated play fighting before the onset of puberty, a "playful" play fighting around mid-puberty, and a "serious" play fighting again in late puberty (Pellis, 2002). These three stages can be easily distinguished based on defensive postures. Before the onset of puberty, between P-15 to P-25, infant rats stand upright and use their forepaws to block the attackers. This type of defense leads to "serious" interactions (Pellis and Pellis, 1997, Pellis, 2002). In mid-puberty, juvenile rats start to rotate their whole bodies to a supine position and use all limbs to block their attackers. This defensive posture makes it easier for the attacker to stand over the partner and to make further attempts to contact the nape, which leads to "more playful" interactions (Pellis and Pellis, 1997, Pellis, 2002). In late puberty, rats shift back to partial rotation defense, which leads to "serious" interactions again (Pellis, 2002). Eventually, this behavior matures into adult aggression, in which rats directly target the back. However, play fighting in rats does not totally disappear after maturating into adulthood. Rats still perform play fighting in adulthood, although at a lower frequency (Pellis and Pellis, 1990; Smith et al., 1999).

Playful play fighting appears rewarding in rats. While performing this behavior, they emit ultrasonic vocalization in the 50 KHz range (Knutson et al., 1998).

Rats produce 50 KHz vocalizations in anticipation or at the receipt of positive rewards, such as sexual encounters and administrations of drugs of abuse (Barfield et al., 1979; Kuntson et al., 1998; Burgdorf et al., 2001). Juvenile rats also make these vocalizations when they anticipate playing (Knutson et al., 1998). These vocalizations can be evoked in juvenile rats by tickling the nape of the neck (Panksepp and Burgdorf, 2003), which is exactly the place being nuzzled during play fighting in this species. Moreover, some studies showed that play fighting itself can be a reinforcer for maze learning and place-preference conditioning in rats (Humphrey and Einon, 1981; Calcagnetti and Schechter, 1992). Together, these data support the concept that the "playful" play fighting is rewarding.

Play Fighting in Hamsters

Hamsters start initiating play fighting as soon as they are capable of coordinated movements between P-15 and P-20 among littermates (Goldman and Swanson, 1975). After weaning on P-25, hamsters become solitary and territorial. During this period, hamsters start using play fighting to establish dominant/subordinate hierarchies and to defend their territories. At this age, play fighting is mainly initiated by the dominant hamster whereas the subordinate hamster avoids contact with the dominant (Delville et al., 2003). The frequency of play-fighting attacks dramatically increases after weaning (Goldman and Swanson, 1975; Pellis and Pellis, 1988b; Wommack et al., 2003). It peaks in early puberty around P-

35 (Wommack et al., 2003). Afterwards, the frequency gradually decreases during puberty and play fighting gradually transforms into adult aggression. When reaching adulthood on P-70, hamsters perform only adult aggression and play fighting totally disappears (Wommack et al., 2003). Compared with rats, play fighting in hamsters is limited to "serious" play fighting.

Development of Agonistic Behaviors in Hamsters

In hamsters, play fighting gradually matures into adult aggression during puberty (Wommack et al., 2003). The difference between play fighting and adult aggression can be described qualitatively and quantitatively. Qualitatively, play fighting and adult aggression target different areas. In this species, play-fighting attacks are targeted at the face and cheeks, while adult attacks are focused on the lower belly and rump (Pellis and Pellis, 1988a; 1988b; Wommack et al., 2003). In early puberty around P-35, most attacks are oriented at the face of the opponents. The proportion of the frontal attacks decreases steadily until P-54 and totally disappears after adulthood on P-70. In contrast, rear attacks appear around P-40. The proportion of rear attacks steadily increases until adulthood. By P-70, hamsters only perform rear attacks. Quantitatively, juvenile hamsters are more active and perform more attacks during agonistic contacts than adults (Wommack et al., 2003; Taravosh-Lahn and Delville, 2004; Cervantes et al., 2007). Attacks are more repetitive in juveniles than in

adults, leading to a higher number of attacks per bout of contact (Cervantes et al, 2007). Moreover, contact bouts are longer and more frequent in juveniles

In hamsters, both play fighting and adult aggression contain offensive and defensive components (Pellis, 2002; Delville et al., 2005). The offensive component relates to behaviors associated with the initiation of attacks, whereas defensive component is performed in response to attacks. In these studies, I used the resident/intruder model using smaller and younger intruders to promote offensive responses by my study subjects (Blanchard and Blanchard, 1988). In hamsters, the size of the protagonists predicts the outcome of the encounters (Drickamer et al., 1973; Delville et al., 2003; Wommack et al., 2003).

Neural Circuitry of Play Fighting in Rats

The neural circuitry of playful play fighting in juvenile rats has been identified by *c*-Fos mRNA in situ hybridization mapping (Gordon et al., 2002). In this study, rats were sacrificed in early puberty (P-32 to P-33) after performing "playful" play fighting. The results showed enhanced *c*-Fos mRNA expression in the parietal cortex, dorsal striatum, ventral striatum, inferior colliculus, deep tectum, dorsolateral tectum, and dorsal periaqueductal gray. In addition, lesion studies indicate that damage to the ventromedial hypothalamus, the anterior hypothalamus, the cortical and the central amygdala inhibited playful play fighting in juvenile rats (Beatty and Costello, 1983; Panksepp et al., 1984; Meaney et al., 1981). Moreover, large lesions of the septum

were associated with enhanced playful play fighting (Panksepp et al., 1984). The brain areas associated with play fighting in rats include the cortex. Lesions of the prefrontal cortex (and in many cases extending well beyond that area) in neonates result in affecting age-related modulation of defensive behavior in play fighting (Pellis et al., 2006). Of course these studies all refer to playful play fighting and may not be particularly relevant to serious play fighting in hamsters, even though the lesions of the amygdala and the septum produce effects similar to those affecting adult aggression. Nevertheless, it is possible that play fighting involves a variety of limbic and cortical regions in hamsters.

Neurotransmitters Controlling Play Fighting in Rats

Few neurotransmitter systems have been associated with play fighting in rats. Though a few studies have pointed to a possible role for dopamine (DA), endogenous opiates, and serotonin (5-HT). Unfortunately, most of these studies involved peripheral treatments of animals and did not point to specific brain areas. For instance, a high dose of a DA agonist, Apomorphine, facilitates play fighting, whereas a low dose of Apomorphine inhibits this behavior (Beatty et al., 1984; Niesink and Van Ree, 1989). In addition, play fighting could be facilitated by an Opioid agonist and inhibited by an Opioid antagonist (Niesink and Van Ree, 1989).

Neural Control of Agonistic Behaviors in Hamsters

Most studies of neural control of agonistic behaviors in hamsters focus on aggressive behavior in adults. One recent study, however, showed that a peripheral injection of high doses of 5-HT reuptake inhibitor, fluoxetine, inhibited play fighting in juvenile hamsters as it does in adult hamsters (Taravosh-Lahn et al., 2006). These results indicate that play fighting and adult aggression in hamsters are mediated by the same neurotransmitters and can be controlled by a common neural circuitry. Below, the neural circuitry and neurotransmitters systems associated with adult aggressions in hamsters are discussed.

Neural Circuitry of Aggression in Hamsters

The anterior hypothalamus (AH) is at the center of a network mediating agonistic behaviors, including aggression and flank marking, in hamsters (Bamshad and Albers, 1996; Ferris et al., 1990; Delville et al., 2000). Microinjections of AVP into the AH facilitates offensive aggression in hamsters in a resident/intruder paradigm (Ferris et al., 1997). Microinjections of an AVP-V1A receptor antagonist into the AH inhibit offensive aggression by resident hamsters (Ferris and Potegal, 1988).

The reciprocal connections between the AH and other brain areas have been identified in hamsters (Ferris et al., 1990; Coolen and Wood, 1998; Delville et al., 2000). The main areas that have reciprocal connections with the AH include the diagonal band of Broca (DBB), lateral septum (LS), medial preoptic area (mPOA), central amygdala (CeA), medial amygdala (MeA), ventrolateral hypothalamus (VLH),

bed nucleus of the stria terminalis (BST), and dorsal lateral part of the midbrain central gray (dlCG). The functional significance of these areas has been identified in the same study through *c*-Fos immunostaining. An enhanced *c*-Fos staining has been found in the MeA, the BST, the VLH, and the dlCG in residential hamsters after attacking intruders (Delville et al., 2000). This result indicates that these areas work together in a network mediating offensive aggression in adult hamsters.

Neurotransmitters Controlling Aggression in Hamsters

Several neurotransmitter systems have been associated with aggression in rodents (Nelson and Chiavegatto, 2001). The followings have been associated with aggression in hamsters. They include vasopressin (AVP), serotonin (5-HT), and CRH.

Vasopressin

In hamsters, microinjections of AVP into the anterior hypothalamus (AH) facilitated offensive aggression (Ferris et al., 1997). This treatment reduced the latency of the resident to bite the intruder and increased the total number of bites. In contrast, microinjections of an AVP V1A-receptor antagonist into the AH caused a dose-dependent inhibition of offensive aggression of a resident male toward an intruder (Ferris and Potegal, 1988). The facilitative effect of AVP on offensive aggression is not limited to the AH. Microinjections of AVP into the ventrolateral hypothalamus (VLH) also facilitated offensive aggression (Delville et al, 1996).

Together, these results indicate that AVP is able to modulate offensive aggression in several brain areas in hamsters, which suggest the existence of a circuitry of vasopressin-sensitive areas controlling agonistic behaviors in hamsters (Ferris et al., 1994).

In hamsters, AVP neurons were mostly restricted in the hypothalamus (Ferris et al., 1995; Delville et al., 1998), including the supraoptic nucleus (SON), the paraventricular hypothalamic nucleus (PVN), the nucleus circularis (NC), and the suprachiasmatic nucleus (SCN). Lesion studies indicate that AVP neurons located in the NC and the medial division of the supraoptic nucleus (mSON) were associated with agonistic behaviors in hamsters (Ferris et al., 1995; Delville et al., 1998). These results were supported by the observation of enhanced *c*-Fos-immunolabeling activity within the same AVP cell groups in adult hamsters after performing offensive aggression (Delville et al., 2000).

Serotonin

As AVP facilitates offensive aggression, another neurotransmitter, serotonin (5-HT), inhibits offensive aggression in adult hamsters. In hamsters, the facilitating effects of AVP at the AH and the VLH can be blocked by peripheral injections of 5-HT reuptake inhibitor, fluoxetine (Delville et al., 1996; Ferris et al., 1997). An *in vitro* receptor autoradiography study showed that the AH contains both AVP and 5-HT receptors (Ferris et al., 1997), which indicates that the AH is a possible targeted area

where the 5-HT work to inhibit aggression. A further study supported this hypothesis by identifying that AVP V1A, 5-HT-1A, and 5-HT-1B receptors are present in the AH (Ferris et al., 1999). Furthermore, the same study also showed that microinjections of a 5-HT-1A receptor agonist into the AH inhibit an AVP-facilitated offensive aggression. The locations of the 5-HT neurons projecting to the AH have been identified through a retrograde tracing (Ferris et al., 1999). The primary sources of the 5-HT innervations to the AH come from the 5-HT neurons in the dorsal and medial raphe nuclei in hamsters (Delville et al., 2000).

Serotonin also mediates play fighting in juvenile hamsters. A recent study showed that the 5-HT reuptake inhibitor, fluoxetine, affected play fighting in juvenile hamsters in early puberty using a resident/intruder paradigm (Taravosh-Lahn et al., 2006). A peripheral injection of high doses of fluoxetine reduced the frequency of attacks and the latency of attacks of the resident hamsters while low doses facilitate the frequency of attacks. This finding indicates that adult aggression and play fighting may be controlled by a similar neural circuitry and mediated by the same neurotransmitters. The facilitative effect of a moderate dose of fluoxetine may be due to the late maturation of the 5-HT system during puberty. The density of 5-HT innervations in the AH is 20% higher in adult hamsters compared with juveniles. The developing 5-HT system in early puberty may be less responsive to the 5-HT, which leads to the results that only high doses of fluoxetine can inhibit offensive aggression in juvenile hamsters. This result shows that 5-HT is involved in modulating both play

fighting and adult aggression in hamsters. Based on this finding, it is possible that AVP, which facilitates offensive aggression in adult hamsters, may also modulate play fighting in juveniles.

Corticotropin-Releasing Hormone

In hamsters, Corticotropin-Releasing Hormone immunoreactive (CRH-ir) fibers and neurons were widely distributed in the brain (Delville et al., 1992). Because CRH-ir cells in hamsters were mainly located within the limbic system, including the medial preoptic area (mPOA), the paraventricular nucleus (PVN), and the central amygdala (CeA), it was possible that CRH is involved in regulating limbic functions, such as social behaviors or autonomic regulations (Delville et al., 1992). Treatment of animals with a oral administration of a CRH1 receptor antagonist resulted in a higher latency to bite an intruder and a lower frequency of attacks (Farrokhi et al., 2004). However, in that study, the treatment not only inhibited the attacks, but also decreased the total contact time. This result indicates that CRH has a more general control in aggression affecting not just consummatory aspects but also possibly appetitive components as well. This result indicates that CRH controls offensive aggression in hamsters, even though the brain area targeted by CRH remains unknown. Based on this finding, it is possible that CRH also facilitates play fighting in juvenile hamsters.

Experimental Overview

Despite the play fighting behavior in juvenile hamsters has been well documented, the neural mechanisms underlying this behavior are poorly understood. One question remained unanswered is: are play fighting and adult aggression are controlled by a similar neural mechanism in hamsters? It is possible that play fighting gradually matures into adult aggression and a common neural mechanism controls both behaviors in hamsters (Delville et al., 2003 and 2005). This possibility is based on the similar components of these two agonistic behaviors. First, the offensive components of both behaviors are initiated by the residents and both behavior sequences have an approach immediately followed by an attempt to bite. Second, juvenile hamsters perform flank marking immediately after a successful attack similar to adult hamsters.

I hypothesize that a common neural system controls both play fighting and adult aggression in hamsters. This hypothesis is tested and the results are organized into several chapters in my dissertation. In Chapter 2, the neural circuitry of play fighting is reviewed. Chapter 3 is dedicated to the discussion of vasopressin and play fighting. In Chapter 4, CRH and its regulation on play fighting is addressed. In Chapter 5, a general discussion of the neural circuitry of play fighting is given, and how this behavior is differently facilitated by vasopressin and CRH is examined in detail.

Chapter 2: Neural Circuitry of Play Fighting

Introduction

Play fighting, a form of agonistic behavior, is common in juvenile mammals and typically performed around puberty before adult aggressive behavior (Vanderschuren et al., 1997; Blanchard et al., 2003; Delville et al., 2005; Pellis, 2002). Play fighting also differs greatly between species, first, in levels of complexity, from complex in rats, including sophisticated attack and defense repertoires between players, to simple in mice, limited to only chasing and evasion (Pellis et al., 1989; Pellis and Pasztor, 1999). In addition, play fighting is not a unitary behavior. As in aggression, play fighting includes offensive and defensive components as animals (Pellis, 2002; Delville et al., 2005). Second, two main types of play fighting have been described during puberty: "playful" and "serious" (Pellis and Pellis, 1998; Pellis, 2002). The former appears to be a rewarding behavior for both participants whereas the latter is used to establish dominant/subordinate hierarchies (Pellis, 1988; Panksepp and Burgdorf, 2003; Delville et al., 2005). Both behaviors are easily observed in a laboratory setting, as rats engage constantly in "playful" play fighting in early puberty around postnatal day 35 (P-35) (Pellis and Pellis, 1987, 1997; Bolles and Woods, 1964; Panksepp, 1981). Later, their behavior is replaced by "serious" play fighting before maturing to adult aggression (Pellis and Pellis, 1997; Pellis, 2002; Foroud and Pellis, 2003). The relative importance of these different forms of play fighting may

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also differ between species during specific developmental period. In golden hamsters, play fighting is limited to "serious" interactions during puberty (Delville et al., 2003). In this species, play fighting peaks in early puberty around P-35 and gradually matures into adult aggression in late puberty (Goldman and Swanson, 1975; Wommack et al, 2003). The "serious" play fighting of hamsters differs substantially from adult aggression quantitatively and quantitatively. Qualitatively, these two behaviors have different target areas. Play fighting attacks of juvenile hamsters are targeted at the face of the protagonist, while attacks by adults are focused on the lower belly and rump (Pellis and Pellis, 1988a; 1988b; Wommack et al., 2003). Quantitatively, juvenile hamsters are more active and perform many more attacks during agonistic contacts than adults (Wommack et al., 2003; Taravosh-Lahn and Delville, 2004). In particular, attacks and bouts of contact are much more repetitive in play fighting than in adult aggression (Cervantes et al., 2006).

Few studies have attempted to identify neural sites associated with play fighting behavior in animals. For instance, in rats, one study showed brain areas activated during "playful" play fighting through changes in *c*-Fos expression in the sub-nuclei of the striatum and the tectum, the inferior colliculus, the dorsal midbrain central gray, and the parietal zone of the somatosensory cortex (Gordon et al., 2002). However, the neural structures associated with "serious" play fighting remain unknown. Based on behavioral similarities between the offensive components of "serious" play fighting and adult offensive aggression, it is possible that a single

neural circuitry mediates the activation of both behaviors (Delville et al., 2003). In adult hamsters, brain areas associated with offensive aggression include the posterior dorsal part of the medial amygdala (MePD), the bed nucleus of the stria terminalis (BST), the ventrolateral hypothalamus (VLH), and the dorsal lateral part of the midbrain central gray (CGdl) (Delville et al., 2000). These areas show enhanced *c*-Fos expression after the performance of offensive aggression. It is hypothesized that similar brain areas will be activated in juvenile hamsters after consummation of offensive play fighting.

The main goal of this study was to test the possibility that a single neural circuitry is associated with the control of the offensive components of "serious" play fighting and adult aggression. This possibility was tested through immunolabeling of *c*-Fos expression, as a marker of neuronal activity (Morgan and Curran, 1989; Kovács, 1998) in the neural circuitry centered of the AH and within areas previously unexplored in adult hamsters, such as the prefrontal cortex.

Methods

Animals and Treatment

This study was carried out with male golden hamsters (*Mesocricetus auratus*) bred in the laboratory from a colony originating from Harlan Sprague Dawley (Indianapolis, IN, USA). All litters were culled to six pups (4 males, 2 females) by

Postnatal days 7 (P-7). All males were weaned on P-25 and housed individually in plexiglass cages (20 x 33 x 13 cm). Hamsters were housed under a reversed light cycle (14:10 light/dark cycle and lights off at 10:00 h) and received food and water *ad libitum*. Their body weights were measured weekly and recorded to monitor their development. The studies were conducted in early puberty (P-35) around the time of peak play fighting activity in this species (Goldman and Swanson, 1975; Taravosh-Lahn and Delville, 2004; Cervantes *et al*., 2006). All procedures were performed according to National Institutes of Health guidelines approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin and conducted in an AALAC-accredited facility.

Experimental Design

On P-30, male hamsters (n=20) were pre-tested for agonistic behavior for 10 minutes individually in the presence of a 10-20% lighter, younger and unfamiliar male intruder. This resident/intruder procedure favors offensive responses by residents (Delville *et al*., 2003). All animals attacked intruders at least once in this test so no animal was excluded. Then, animals were divided into two comparable groups (n=10 in each) based on the body weight and the agonistic behaviors observed in this test. On P-35, animals in the experimental group were observed for agonistic behaviors during a 10-min encounter with another unfamiliar intruder. Animals in the control group were remained in the presence of a block of wood (5 x 9 x 1.5cm), which had been left overnight in the cage of unfamiliar intruders and carried their odor, for 10

minutes. This control was used to activate neural activity associated with social arousal and to make sure the enhanced neural activity observed in experimental animals was specific to the consummation of play fighting. In previous studies with adults, exposure to the woodblock elicited flank-marking behavior (Delville *et al*., 2000), an element of the ethogram of agonistic behavior in hamsters, but short of its consummation (Siegel, 1985). Agonistic encounters of animals were videotaped in both groups for later review. After 50 minutes, all animals were deeply anesthetized with an injection of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL; 100 mg/kg, IP) and were perfused transcardially. The perfusion procedure started with 0.9% saline containing 0.2% sodium nitrite for 2 minutes to dilate the blood vessels, and then followed by a fixative solution containing 4% paraformaldehyde and 2.5% acrolein in 0.1M potassium phosphate buffered saline (KPBS, pH 7.4) for 18 minutes, and then followed by saline for another 2 minutes to wash out residual fixative solution. Afterwards, brains were removed from the skulls and placed in 20% sucrose in KPBS at 4^0C for at least 24 hours. Then, brains were sliced into 40 μm-thick coronal sections with a freezing rotatory microtome. Brain sections were saved in a cryoprotectant (Watson et al., 1986) at -20^0C until labeled by immunocytochemistry.

Behavior Observations

During the 10-minute resident/intruder encounters, a number of agonistic behaviors performed by residents were observed, including attack, pin, and flank marking. Attacks were defined as a combination of an approach immediately followed by an attempt to bite (Wommack *et al*., 2003). Pins were defined as one animal lying on its back with the other animal on top. Flank marking was identified when hamsters rubbed their flank glands on the walls of cages (Johnston, 1985), which was usually performed by residents after successful attacks.

***c*-Fos Immunocytochemistry**

Brain sections were processed for immunocytochemistry to *c*-Fos as described in a previous study (Delville et al., 2000). First, brain sections were washed in 0.05M KPBS buffer to remove the cryoprotectant, and the sections were treated with 1% sodium borohydrate in KPBS for 10 minutes to remove the residual aldehydes. After several washes, brain sections were pre-incubated in a KPBS solution with 20% normal goat serum (NGS) to prevent non-specific labeling, 1% hydrogen peroxide to eliminate unreacted peroxidase in the blood vessels, and 0.3% Triton X-100 to make the sections permeable. Then, the sections were incubated for 48 hours at 4ºC with a rabbit polyclonal primary antibody to *c*-Fo*s* (0.05µg/ml, sc-52, Lot H024, Santa Cruz Biotechnology Inc., Santa Cruz, CA) recognizing an sequence (residues 1-16) at the N-terminus of *c*-Fos of human origin (Finkel et al., 1966; Nishizawa et al., 1987) in

"KPBS wash" solution (0.05M KPBS with the presence of 2% NGS and 0.3% Triton X-100). After several washes, the sections were successively incubated for 45 minutes at room temperature in a secondary antibody (biotinylated goat anti-rabbit IgG, 2.5 μg/ml, Lot T0411, Vector Laboratories, Inc., Burlingame, CA) in "KPBS wash" solution. After several rinses, the sections were placed for 45 minutes at room temperature in a tertiary incubation with an avidin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories, Inc.) to form a horseradish peroxidase (HRP) complex bound to the primary/secondary antibodies complex. After several more rinses, the sections were labeled with nickel-conjugated diaminobenzidine (nickel sulfate 2.5%; diaminobenzidine 0.02%; hydrogen peroxide 0.0025%) in 0.175M sodium acetate buffer (pH 5.8) to form a blue-black precipitate over the nuclei of stained cells. This immunocytochemical procedure was previously validated in hamsters through omission of the primary antibody and pre-incubation of the primary antibody with purified control peptide (Delville et al., 2000).

Quantification of Immunocytochemistry

All slices of brain sections were coded randomly to ensure blind measuring. Images were digitized through a video camera connected with an Apple computer through a frame grabber. The illumination was kept constant for all measurements. A gray-scale normalization was performed to minimize background variations of all images (Delville et al., 2000). *c*-Fos-immunoreactive (*c*-Fos-ir) cells were counted

within a square of 250x250 μm placed on the areas of interest using NIH Image (v. 1.62, NIH, Bethesda) after gray-scale thresholding. Manual verifications were performed to prevent mistakes from multiple cells counted as one by the software.

Areas selected for quantification included the AH, areas with reciprocal connections with the AH (Delville et al., 2000), the prefrontal cortex (PFC), and the anterior parietal cortex (APC) (Fig. 2.1). The AH is associated with mediating offensive aggression in hamsters (Ferris et al., 1997). Areas reciprocally connected to the AH and selected for quantifications included the lateral edge of the lateral septum (LS), the posterior part of the medial division of the bed nucleus of the stria terminalis (BST), the medial part of the preoptic area (POA), the ventrolateral part of the ventromedial hypothalamus (VLH), the posterodorsal part of the medial amygdaloid nucleus (MePD), the central amygdaloid nucleus (CeA), and the dorsolateral part of the midbrain central gray (CGdl). Most of these areas show enhanced activity after performing offensive aggression in hamsters (Delville et al., 2000). Selections of the PFC included the medial part (mainly layers III- IV cells) of the cingulate cortex (Cg1), the prelimbic cortex (PrL), and the imfralimbic cortex (IL). The PFC has been shown to be associated with aggressive behavior in humans (for review, Bufkin and Luttrell, 2005) and animals (Haller et al., 2006; Halász et al., 2006). The APC is part of the somatosensory cortex receiving inputs from the vibrissae, which is associated with attack and aggression, as well as play fighting (Ahl, 1986; Gordon et al., 2002).

In current study, the counting square mainly covered layer III and layer IV cells of the APC. The locations where the 250 μm^2 counting squares were placed in these quantified areas were illustrated in sections drawn with camera lucida in Figure 2.1. The landmarks of all areas quantified were based on Nissl-stained sections previously used by the Dr. Delville Lab. Two example pictures of Nissl-stained sections from animals at the same age (P-35) indicating the exact locations where the counting squares were placed in the VLH and the MePD were shown in Fig. 2.2 (right column). For each area, 4-7 consecutive sections were measured bilaterally in each animal (8-14 measures per animal). These measures were averaged for each individual, and the averages were compared between groups.

Data Analysis

All anatomical measures were compared between groups with independent Student t-tests (two-tailed) for each area sampled. Correlation coefficients were calculated between each area for each group independently to identify the functional associations of brain areas mediating behaviors. A jackknife procedure was performed to ensure the reliability of correlations. Each individual subject's data was dropped from a group, and the correlations were calculated again without this subject. This procedure continued until each subject in one group had been dropped and correlations were re-calculated. If the significance of a correlation disappeared more than once in the re-calculations, it was considered unreliable and not reported to be significant in this study. The same method with similar criteria has been used to build

functional networks of latent inhibition learning in mice (Puga et al., 2007). Each correlation coefficient was converted into a Z-value through the Fisher Z transformation (Hays, 1994). These Z-values were compared between groups for each correlation between areas using the following formula:

$$Z = \frac{(Z_{ij1} - Z_{ij2})}{\sqrt{[[1/(n_1 - 3)] + [1/(n_2 - 3)]]}}$$

In this formula, Z_{ij} is the Fisher Z-transformed value for the correlation coefficient between areas 'i' and 'j', while n_1 and n_2 are numbers of animals in group 1 (experimental) and group 2 (control) respectively. These comparisons were used to identify significant changes ($P<0.05$) in correlations of different brain areas between different behavioral conditions (performing play-fighting attacks versus arousal state induced by olfactory stimulus), as previously explained for pCREB-ir cells in relation to offensive aggression and emotional reactivity (David et al., 2004). Similar correlation analysis has also been used for the expression of immediate early gene products and the autoradiographic mapping in different species (Hoke et al., 2004; Jones and Gonzalez-Lima, 2001).

Results

Behaviors

Hamsters in the experimental group (n=10) initiated olfactory investigations of the intruders, and then attacked, pinned and bit their intruders several times. The

median number of attacks performed by residents was 11 (range 1 to 15), and the median number of pins was 15 (range 2 to 19) in this experiment. The residents also flank marked occasionally during the encounters. All control animals exposed to the woodblock (n=10) performed olfactory investigations. Some hamsters flank marked their cage and nibbled the woodblocks.

_c_-Fos Quantifications

In this study, *c*-Fos-ir cells were found in every area selected for quantification (degree of freedom = 18) (Figs. 2.2 and 2.3). In some areas, no statistically significant difference was found between experimental and control groups. These areas included the AH ($p=0.086$), the CeA ($p=0.44$), the POA ($p=0.2$), and the CGdl ($p=0.25$). In other limbic areas, the density of *c*-Fos-ir cells was 25 to 50% greater in experimental animals as compared to controls. These areas included the VLH ($p<0.001$), the MePD ($p<0.01$), the BST ($p<0.0001$), and the LS ($p<0.05$). In the cortex, all prefrontal cortical areas showed a statistically significant 20% greater density of *c*-Fos-ir cells in experimental animals (Cg1, $p<0.01$; PrL, $p<0.01$; IL, $p<0.05$] (Fig. 2.4). However, no statistically significant difference was observed between experimental and control groups in the APC ($p=0.18$).

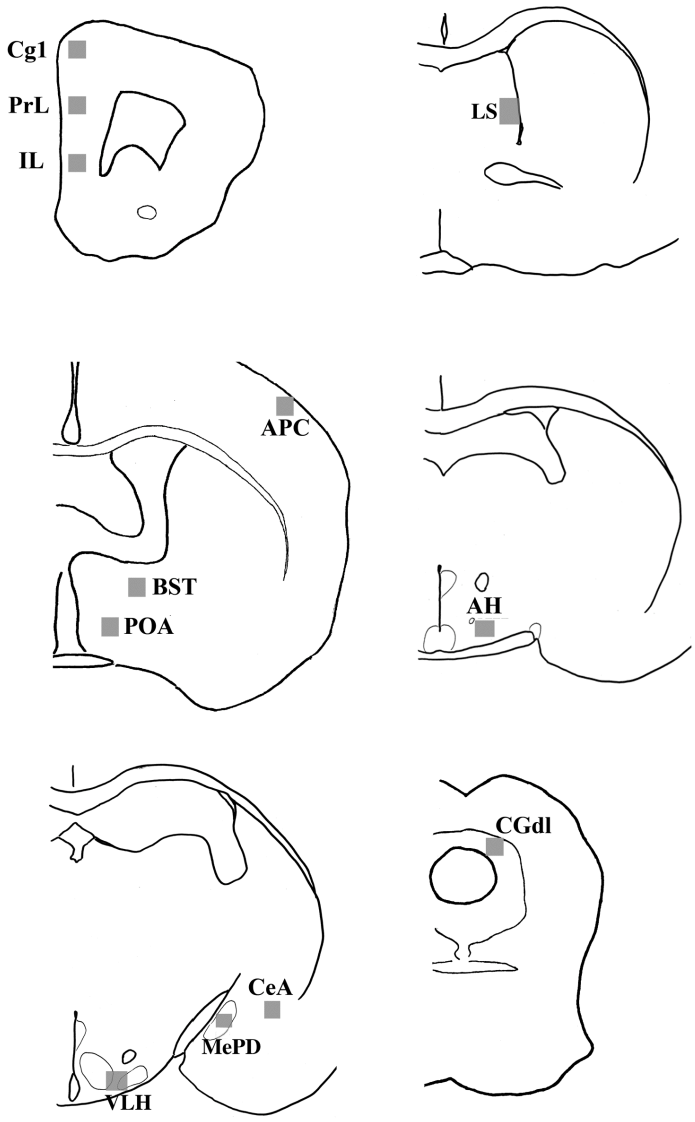
Correlation Analyses

Correlations were performed between brain areas within groups (Table 2.1). In control animals, statistically significant correlations were found between the AH and the prefrontal cortical areas (AH-PrL: $r=0.862$, $p<0.01$; AH-IL: $r=0.764$, $p<0.05$; AH-

Cg1: $r=0.813$, $p<0.05$). In addition, a significant correlation was found between areas within the PFC (IL-PrL: $r=0.886$, $p<0.01$). In experimental animals, statistically significant correlations were found between two areas in the PFC (PrL-IL: $r=0.827$, $p<0.01$), and between the CGdl and the LS (CGdl-LS: $r=-0.821$, $p<0.01$).

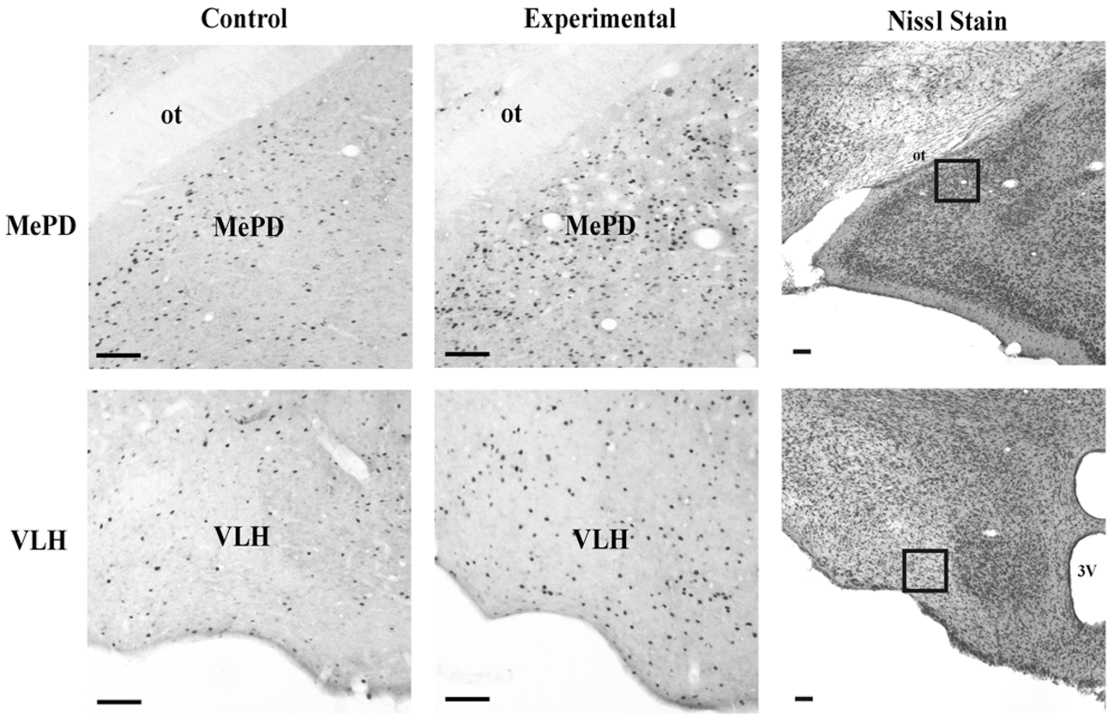
Comparisons of correlation coefficients were performed for each pair of areas between experimental and control groups (Table 2.2). A significant decrease in correlation was found between the AH and the Cg1 between control and experimental groups ($Z=-2.22$, $p<0.05$). Furthermore, significant decreases in correlations between groups were also found between the LS and the CGdl ($Z=-2.45$, $p<0.05$).

Figure 2.1: the locations of the brain areas selected for *c*-Fos-ir quantification.



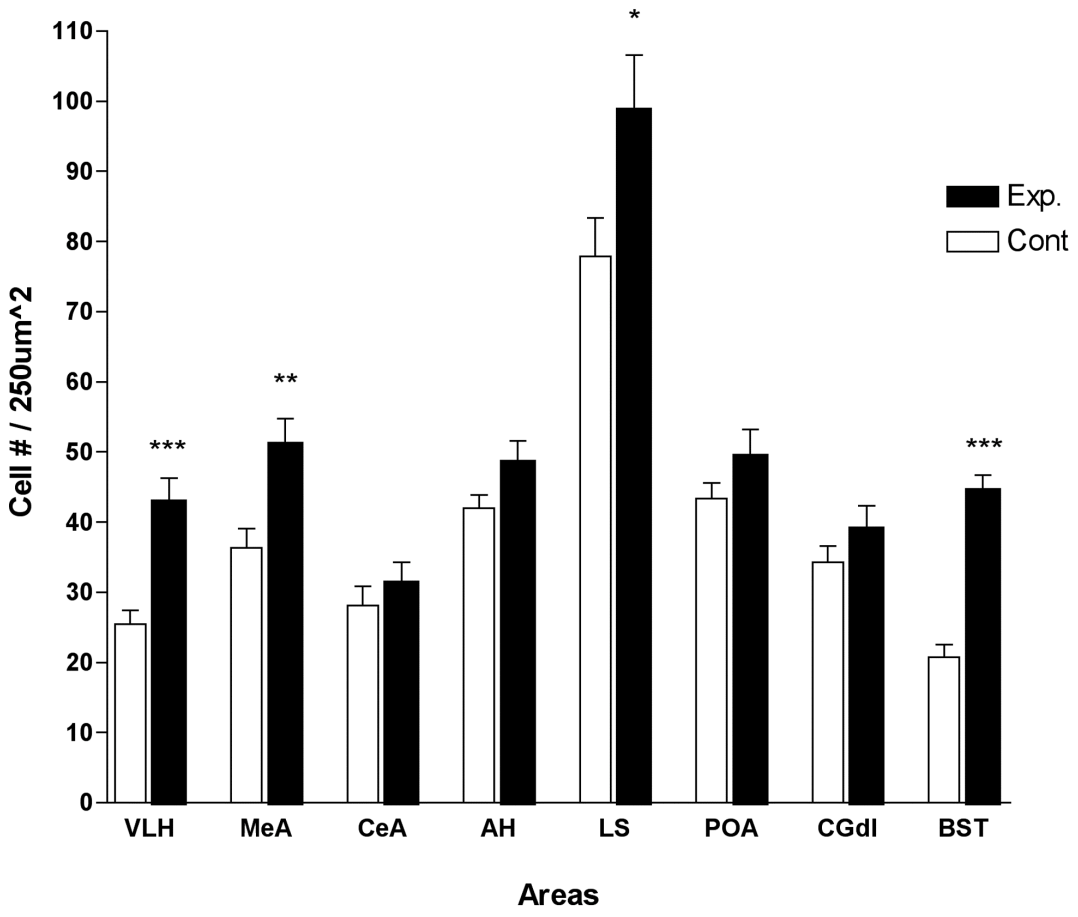
Diagrams indicating the locations of the brain areas selected for *c*-Fos-ir quantification. Areas include the prelimbic cortex (PrL), the infralimbic cortex (IL), the cingulate cortex (Cg1), the Anterior Pariental Cortex (APC), the ventrolateral hypothalamus (VLH), the posterodorsal part of the medial amygdala (MePD), the central amygdala (CeA), the anterior hypothalamus (AH), the lateral septum (LS), the preoptic area (POA), the dorsolateral part of the midbrain cental gray (CGdl), and the bed nucleus of the stria terminalis (BST). Representative coronal sections of the areas of interest were drawn using a camera lucida attachment on a microscope. The grey square indicates the locations of the 250 μm^2 counting square placed in these brain areas.

Figure 2.2: examples of c-Fos and Nissl staining pictures



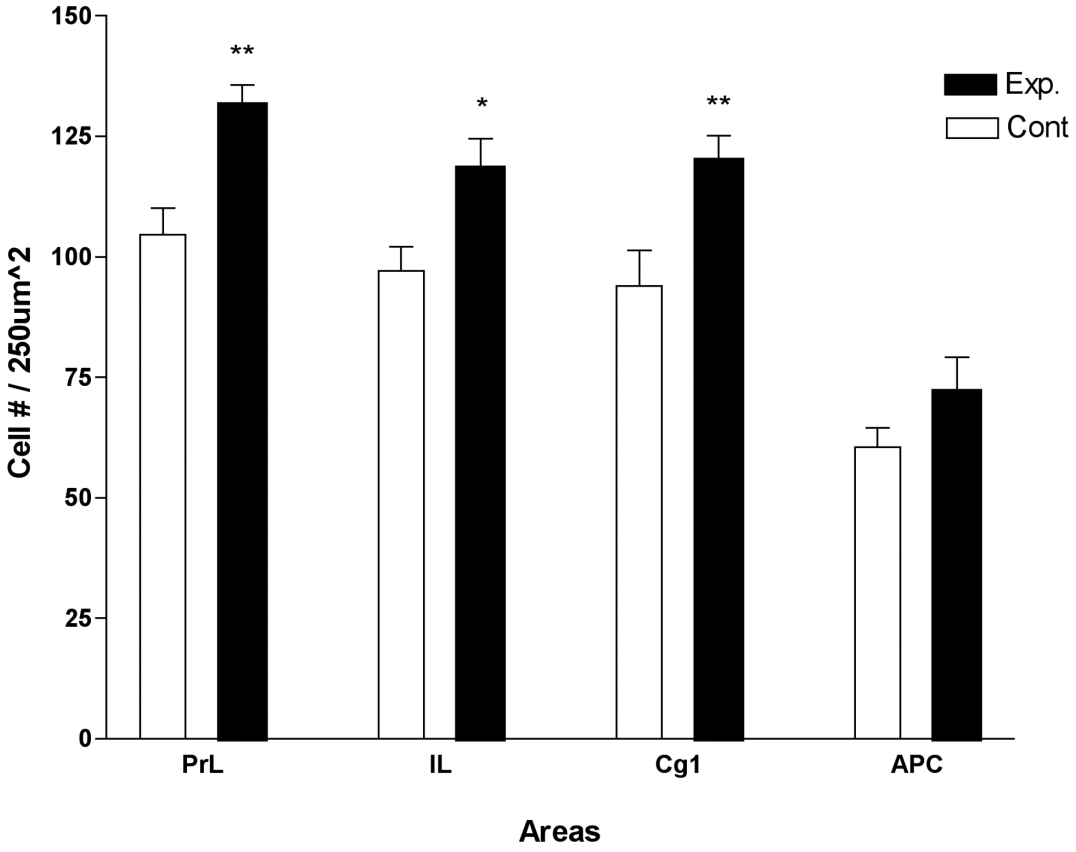
Photomicrographs showing *c*-Fos-ir cells in the the posterodorsal part of the medial amygdala (MePD), and the ventrolateral hypothalamus (VLH) in sections from representative animals in the control (left column) and the experimental (middle column) groups, and Nissl-stained (right column) sections from animals at the same age (P-35) showing the exact locations of the 250 μm^2 counting squares. Ot: optic tract. 3V: third ventricle. Scale bars = 100μm.

Figure 2.3: c-Fos expression in limbic areas



Comparison of the *c*-Fos-ir labeled cell density between control animals (exposed to a woodblock, n=10) and experimental animals (attacking an intruder, n=10) in areas which have reciprocal connections with the anterior hypothalamus. Areas include the ventrolateral hypothalamus (VLH), the posterodorsal part of the medial amygdala (MePD), the central amygdala (CeA), the anterior hypothalamus (AH), the lateral septum (LS), the preoptic area (POA), the dorsolateral part of the midbrain cental gray (CGdl), and the bed nucleus of the stria terminalis (BST). *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$ (Student t-test).

Figure 2.4: c-Fos expression in cortical areas



Comparison of the *c*-Fos-ir labeled cell density between control animals (exposed to a woodblock, n=10) and experimental animals (attacking an intruder, n=10) in the prefrontal cortical areas, which include the prelimbic cortex (PrL), the infralimbic cortex (IL), and the cingulate cortex (Cg1), and the Anterior Pariental Cortex (APC).
*= $p<0.05$; **= $p<0.01$ (Student t-test).

Table 2.1: correlations of c-Fos expression in brain areas in control and experimental animals

		VLH	MEPD	CEA	AH	LS	POA	CGDL	BST	PRL	IL	CG1	APC
Control													
	VLH	1	0.564	0.549	0.343	0.196	0.648	0.432	0.567	0.147	0.411	0.243	-0.281
	MEPD		1	0.676	0.56	0.032	0.209	0.351	0.361	0.533	0.599	0.677	-0.163
	CEA			1	0.538	-0.356	0.535	0.574	0.37	0.481	0.389	0.626	0.112
	AH				1	0.194	0.456	0.426	0.561	**0.862	*0.764	*0.813	-0.395
	LS					1	0.103	0.199	0.366	0.122	0.217	0.26	-0.346
	POA						1	0.703	0.619	0.237	0.261	0.136	-0.128
	CGDL							1	0.416	0.104	-0.065	0.468	-0.258
	BST								1	0.675	0.588	0.41	-0.021
	PRL									1	**0.886	0.76	-0.224
	IL										1	0.658	-0.417
	CG1											1	-0.591
	APC												1
	N=8-10 per group												
Experimental													
	VLH	1	0.269	0.624	0.633	0.233	-0.126	-0.354	0.613	0.427	0.171	-0.146	-0.193
	MEPD		1	0.505	0.642	0.506	0.465	-0.3	0.474	0.279	0.404	0.376	-0.242
	CEA			1	0.635	0.391	0.281	-0.245	0.667	0.742	0.453	0.528	0.161
	AH				1	0.615	0.56	-0.415	0.519	0.171	-0.078	-0.207	-0.564
	LS					1	0.457	**-0.821	0.017	0.422	0.235	-0.139	-0.557
	POA						1	-0.237	0.129	0.132	0.179	0.102	-0.448
	CGDL							1	-0.171	-0.489	-0.318	0.385	0.35
	BST								1	0.547	0.365	0.302	0.273
	PRL									1	**0.827	0.424	0.279
	IL										1	0.618	0.244
	CG1											1	0.569
	APC												1
	N=9-10 per group												

Correlations of *c*-Fos-ir cell density between brain areas in control animals (n=8 in PrL, IL, and Cg1; n=10 in all others) and in experimental animals (n=9 in PrL, IL, Cg1, and CGdl; n=10 in all others). The bold letters represent the statistically significant correlations ($p<0.05$). *= $p<0.05$; **= $p<0.01$.

Table 2.2: changes in correlations of c-Fos expression between control and experimental groups

		VLH	MEPD	CEA	AH	LS	POA	CGDL	BST	PRL	IL	CG1	APC
Control vs Experimental groups													
	VLH												
	MEPD												
	CEA												
	AH									-1.86	-1.79	***-2.22**	
	LS							***-2.45**					
	POA												
	CGDL												
	BST												
	PRL										-0.37		
	IL												
	CG1												
	APC												
	N=8-10 per group												

Comparisons of correlations of *c*-Fos-ir cell density between control and experimental groups. The bold letters represent the statistically significant correlations ($p<0.05$). *= $p<0.05$.

Discussion

It has been hypothesized that a single core neural circuitry controls agonistic behavior throughout development in hamsters (Delville et al., 2003). I predicted that brain areas activated in juvenile hamsters after performing play fighting would be similar to those observed in adult hamsters after offensive aggression (Delville et al., 2000). I found that similar areas, including the VLH, the MePD, and the BST, activated after offensive aggression in adults were also activated after offensive play fighting in juveniles. This finding supports my hypothesis that the offensive components of play fighting and adult aggression in hamsters are controlled by a common neural circuitry.

In juvenile rats, a large increase in *c*-Fos mRNA expression was found in the dorsolateral tectum and the dorsal and ventral striatum after playful play fighting (Gordon et al., 2002). Other areas showing significant increase of *c*-Fos expression in that study included the inferior colliculus, the dorsal midbrain central gray, and the parietal zone of the somatosensory cortex. The activated pattern was very different from what been reported in adult rats after aggression. In adult rats, an enhanced *c*-Fos expression was found in the BST, in the amygdala (medial, caudal, and anterior parts), in the hypothalamus (anterior, dorsomedial, and ventrolateral areas), and in the brainstem (dorsal periaqueductal grey) in residents after performing offensive aggression to intruders (Veening et al., 2005). These findings indicate that playful play fighting and adult aggression may be controlled by different neural circuitries in

rats. In my current finding, offensive component in serious play fighting in juvenile hamsters enhance neural activity in the VLH, the MePD, the BST, and the LS. Similar limbic areas have also been reported in adult hamsters after offensive aggression (Delville et al., 2000). These findings indicates that play fighting and adult aggression in hamsters are controlled by a common neural circuitry. The different findings in rats and hamsters may result from the behavioral differences in these two species. Playful play fighting in juvenile rats does not appear to carry an immediate biological benefit for both protagonists (Pellis, 1988; Panksepp and Burgdorf, 2003). In contrast, play fighting in juvenile hamsters is mainly used to establish dominant/subordinate hierarchies (Pellis and Pellis, 1988a, b). These results indicate that the neural circuits associated with serious play fighting in hamsters are closer to aggression than play.

The nuclei in the limbic areas associated with offensive play fighting in juvenile hamsters were identified through the *c*-Fos immunocytochemistry. The areas included the VLH, the MePD, the BST, and the LS. All four areas were reported to be associated with agonistic behaviors in hamsters or in other species. For example, an enhanced *c*-Fos expression was found in these areas after aggression in hamsters or other rodents (Delville et al., 2000; Haller et al., 2006; Gobrogge et al., 2007). In addition, microinjections of AVP within the VLH and the MePD facilitate offensive aggression in hamsters and rats (Delville et al., 1996; Koolhaas et al., 1990). The BST is generally believed to be an extension of the amygdala (for review, de Olmos and Heimer, 1999). In hamsters, tracing studies show that the medial division of the BST

is strongly interconnected with the MePD (Wood and Swann, 2005). Therefore, it is possible that the BST plays a similar role as the MePD does in modulating play fighting in juvenile hamsters. The LS is involved in the integration of olfactory information in mediating flank marking in hamsters (Irvin et al., 1990). Septal lesions increase aggression in hamsters (Sodetz and Bunnell, 1970). Therefore, it is possible that the LS performs a similar function in mediating play fighting in juvenile hamsters. In current study, the number of *c*-Fos cells in the AH does not differ significantly between groups, though there is a statistical trend ($p=0.09$). This observation should not be interpreted as this area is not involved in play fighting. Furthermore, a similar finding has been reported for this area in adult hamsters under similar testing conditions (Delville et al., 2000). In this case, the presence of the woodblock elicited some flank marking activity, also controlled by the AH (Ferris et al., 1984, 1994), which could prevent differences found between groups. Thus, enhanced labeling in other areas is specific to the consummation of play fighting. But the AH could still play a role on the activation of the initial phase of the ethogram of serious play fighting.

In addition, quantification of *c*-Fos-ir labeling was carried out in the PFC and the APC. The enhanced *c*-Fos-ir labeling found in the PFC indicates that the neural activity in the prefrontal cortical areas, including the PrL, the IL, and the Cg1 is associated with offensive play fighting. Since no significant difference in labeling was found in the APC, it indicates that this difference could not be generalized to all

cortical areas and is specific to the PFC. In human, the PFC inhibits aggression (for review, Bufkin and Luttrell, 2005). It is possible that the PFC has a similar role in hamsters even with increased *c*-Fos expression after aggression. In current study, the selected areas of the PFC for quantification were located in the medial part and mainly in layer III and IV. Therefore, it is possible that the enhanced activity comes from inhibitory inter-neurons indirectly involved in the modulation of play fighting. In rats and mice, enhanced neural activity in the PFC was also found after agonistic contacts (Haller et al., 2006; Halász et al., 2006). It is possible that the orbital frontal cortex may play a similar role as rats with neonatal lesions in this area increased the likelihood of using a rougher defensive position in play fighting (Pellis et al., 2006). Thus, it is possible that though inter-neurons in the prefrontal cortex are activated during the performance of play fighting in hamsters, they modulate a net inhibitory output of the area to the behavior.

The analysis of correlations of *c*-Fos-ir expression within brain areas may support this possibility. In current study, an enhanced *c*-Fos expression was found in several limbic areas and the PFC in experimental animals compared to controls. However, changes in signals between group comparisons do not indicate whether these changes could be found in the same individuals. In addition, it does not provide enough information of functional coordination between brain areas of animals performing different behaviors. Even though a significant correlation does not represent a direct neuro-anatomical connection between brain areas, these results

provide a functional map of activated regions under different behavioral environments. In control animals, the neural activity in all three areas of the PFC (Cg1, PrL, and IL) was significantly correlated with the neural activity in the AH. Interestingly, these significant correlations disappeared entirely in animals that performed attacks, and the change was significant between the Cg1 and the AH. This result suggests that, in juvenile hamsters, play fighting is associated with a de-synchronization of neural activity between the PFC and the AH. This outcome is consistent with an inhibitory role of the PFC on play fighting.

In summary, these data support my hypothesis that a common neural circuitry controls the offensive component of play fighting and adult aggression in hamsters. The elements in this circuitry belong to a social behavior neural network controlling different kinds of social behaviors in vertebrates (Goodson, 2005). This network may control different behaviors through strengthening or de-synchronizing correlations of neural activity in different brain areas in the same network, or from other areas outside of the network, as the PFC. Elements of this network may be modulated by AVP or other neurotransmitter systems to enhance offensive responses. This aspect is addressed in next chapter. The entire system appears to be functional before puberty, though some of its elements may be modulated by the changing endocrine milieu associated with puberty.

Chapter 3: Vasopressin and Play Fighting

Introduction

Play fighting is a juvenile form of agonistic behavior preceding adult aggression (Delville et al., 2005; Pellis, 2002). In hamsters, this behavior is initiated before puberty, peaks in early puberty around postnatal day 35 (P-35) and gradually matures into adult aggression in late puberty (Golden and Swanson, 1975; Wommack et al., 2003). Differing from adult aggression, play fighting is characterized with more repetitive attacks and contact bouts during agonistic interactions than adults (Wommack et al., 2003; Taravosh-Lahn and Delville. 2004; Cervantes et al., 2006). In addition, juvenile hamsters target the face of the protagonist in play fighting, while adult hamsters focus on the lower belly and rump (Wommack et al., 2003; Pellis and Pellis, 1988a; 1988b).

The possibility that vasopressin (AVP) is associated with play fighting is interesting as this peptide plays a key role in the control of offensive aggression in adult hamsters (Ferris and Delville, 1994). Microinjections of AVP into the anterior hypothalamus (AH) facilitate offensive aggression (Ferris et al., 1997). This treatment reduces the latency of the resident to bite the intruder and increases the total number of bites. In contrast, microinjections of an AVP V1A antagonist into the AH inhibit expressions of offensive aggression (Ferris and Potegal, 1988).

* This chapter has been published in Cheng and Delville, 2009.

In hamsters, AVP neurons are primarily located within the supraoptic nucleus (SON), the paraventricular hypothalamic nucleus (PVN), the nucleus circularis (NC), and the suprachiasmatic nucleus (SCN) (Ferris et al., 1995; Delville et al., 1998). Neurons within the NC and the medial division of the supraoptic nucleus (mSON) are the likely sources of AVP involved in the control of aggression. Indeed, agonistic behaviors are inhibited after lesions of the NC and the mSON, but not other areas (Ferris et al., 1995; Delville et al., 1998). In addition, AVP neurons in the same two areas also show enhanced *c*-Fos expression in adult hamsters during the consummation of offensive aggression (Delville et al., 2000).

Whether AVP plays a similar role in offensive play fighting in juvenile hamsters remain unknown. An early study indicates that AVP levels in the hypothalamus increases 2-3 folds between P-18 and P-22 in hamsters (Ferris et al., 1996). This timing corresponds to the onset of play fighting (Goldman and Swanson, 1975). However, no study is dedicated to identifying the development of the AVP system and its role in play fighting in early puberty. Since a common neural circuitry controls the offensive components of both play fighting and adult aggression, it is possible that AVP controls play fighting and the increased intensity of attacks in early puberty is associated with the development of this system. This chapter is dedicated to identify the role of AVP involved in play fighting with three designed experiments.

Methods

Animals and Treatment

The present study was carried out with male golden hamsters (*Mesocricetus auratus*) bred in the laboratory from a colony originating from Harlan Sprague Dawley (Indianapolis, IN, USA). All litters were culled to six pups (4 males, 2 females) by P-7. All males were weaned on P-25 and housed individually in plexiglass cages (20 x 33 x 13 cm). The hamsters were housed under a reversed light cycle (14:10 light/dark cycle and lights off at 10:00 h) and received food and water *ad libitum*. Their body weights were measured weekly and recorded to monitor their developments. The studies were conducted in early puberty between P-28 to P-35 around the peak time of play fighting activity performed in this species (Goldman and Swanson, 1975; Taravosh-Lahn and Delville, 2004). All procedures were performed according to the National Institutes of Health guidelines approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin and conducted in an AALAC-accredited facility. All procedures were optimized for minimizing the number of animals used and the suffering of animals.

Experiment 1: Immunocytochemistry to c-Fos Combined With Vasopressin

Experimental Design

The experimental procedure by using the resident/intruder task and the perfusion procedure were the same as been described in Chapter 2.

Combined Immunocytochemistry

Immunocytochemistry to *c*-Fos combined with AVP was performed to identify the activated AVP cells as described in a previous study in adult hamsters (Delville et al., 2000). Procedures of labeling *c*-Fos were the same as described in Chapter 2, except that NGS was replaced by purified normal donkey serum (NDS) in all procedures and the secondary antibody was a biotinylated donkey-anti-rabbit IgG (9 μg/ml, Lot 72488, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) with the same concentrations. After labeling *c*-Fos, the sections were pre-incubated in 20% NDS, 1% hydrogen peroxide and 0.3% Triton X-100 prepared in a 0.1M phosphate buffered saline (PBS). Then, the sections were incubated for 48 hours at 4ºC with a primary antibody (a mouse monoclonal anti-AVP, BER-312, 1/8000, generously donated by Dr. F. Robert, INSERM, France) recognizing the six amino acid cyclic part of vasopressin (Robert et al., 1985, 1991) prepared in "PBS wash" solution (0.1M PBS containing 2% NDS and 0.3% Triton X-100). After several washes, the sections were incubated for 45 minutes at room temperature with a secondary antibody, a biotinylated donkey anti-mouse IgG (9 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) prepared in "PBS wash" solution. After a 45-minute tertiary incubation with an avidin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories, Inc.) and several more washes, the sections were labeled with diaminobenzidine (0.05% in 0.1M PBS) to form a brown precipitate over the soma of

stained cells. Double-labeled cells can be visualized with a brown soma for AVP and a black nucleus for *c*-Fos expression. The specificity of the AVP antibody was previously tested (Robert et al., 1985) and this procedure was previously validated in hamsters through omissions of the primary antibody and pre-incubation in excess amount of AVP (Delville et al., 2000). The resulting distribution of AVP immuno-reactive (AVP-ir) cells and fibers were consistent with previous observations in hamsters using other antibodies or *in situ* hybridization (Delville et al., 1998; Ferris et al., 1995; 1996).

Quantification of Immunocytochemistry

The proportions of AVP-ir cells also containing *c*-Fos-ir were counted from camera lucida drawings taken consecutive section of the hypothalamus. In the hypothalamus, AVP-ir cells were counted in four areas: the PVN, the NC, the mSON, and the lateral division of the supraoptic nucleus (lSON), as previously described (Ferris et al., 1992; Delville et al., 1994, 2000). In these areas, double-labeled cells were analyzed as the percentage of AVP-ir cells also containing *c*-Fos-ir labeling. Six to ten measures were taken from each individual in each area. These measures were averaged for each individual and compared between groups.

Data Analysis

All anatomical measures were compared between groups with independent Student t-tests (two-tailed) for each area sampled.

Experiment 2: Microinjection of Vasopressin Antagonist into the AH

Experimental Design

On P-30, male hamsters were pre-tested for agonistic behavior for 10 minutes individually in the presence of a smaller (10-20% lighter) and younger unfamiliar male intruder. This resident/intruder procedure favors offensive responses by residents (Delville et al., 2003). Animals performing no attack on intruders were excluded from the current study, which usually occurs one in every fifteen animals. Then, animals were divided into three comparable groups based on their body weight and the agonistic behaviors observed in this test.

On P-35, hamsters (n=52) were anesthetized with isoflurane (3% for onset and 2% for maintaining) and placed in a stereotaxic apparatus. A small incision was made in the skin above the skull. A small hole was drilled into the skull. Microinjections were made through a 33-gauge needle attached to a 1μl Hamilton syringe by PE 20 tubing, which was lowered to the AH through the hole. The animals were injected with either 0μM (n=19), 9μM (n=13), or 90μM (n=20) Manning compound [d$(CH_2)_5$Tyr(Me)AVP, Sigma, St. Louis. MO]), dissolved in saline (100nl). Manning compound is a long-lasting vasopressin V1A-receptor antagonist, which inhibits agonistic behaviors in hamsters for at least 12 hrs in a previous study (Ferris et al., 1988). The dosage used in this experiment had been optimized in previous studies on agonistic behavior in hamsters (Ferris and Potegal, 1988; Delville et al., 1996). The coordinate of the AH for hamsters at P-35 was 1.1mm anterior to bregma, 1.7mm lateral to midline, and 7.6mm down from the dura at an 8^0 angle from the midline.

The incisor bar was leveled at +1.5mm. After the stereotaxic injection, a local anesthesia (2% Xylocaine, Astra USA Inc., Westborough, MA) was applied to the wound before closure. The entire procedure (including anesthesia) took less than 15 minutes. The animals woke up less than 2 minutes after the gas anesthesia was removed and were brought back to the animal room. Three hours later, animals were fully active and were observed for agonistic behaviors during a 10-min encounter with another unfamiliar intruder. Agonistic encounters of animals were videotaped with a digital video camera (Sony Digital 8 Handycam, Sony Corporation of America, New York, NY, USA) for later review. After testing, all animals were sacrificed and their brains were fixed by immersion in 10% Acrolein and then sliced into 40 μm-thick coronal sections with a freezing rotatory microtome. A Nissl stain was performed on these tissues to localize the position of the tip of the 33-gauge microinjection needle. Thirty-two animals with correct injection sites in the AH were included in behavioral analysis (0μM: n=10; 9μM: n=10; 90μM: n=12) (Fig. 3.3). Some animals were injected outside the AH (0μM: n=9; 9μM: n=3; 90μM: n=8) within the medial preoptic area (mPOA) and the ventromedial hypothalamus (VMH). These animals were used for comparison. In addition, a pilot test was performed on a smaller group of animals to determine whether the anesthesia could affect behavioral activity. These animals were tested for play fighting just before anesthesia followed by a sham surgery and again 3 hours later, with no apparent effect.

Behavioral Observations

Agonistic interactions were reviewed with iMovie (Apple Computers, Inc., Cupertino, CA, USA) and quantified with EventMonitor software (courtesy of Dr. J. Alberts, Bloomington, IN, USA) for second-by-second analysis (Fig. 3.4). During the 10-minute resident/intruder encounters, a number of behaviors performed by residents were observed, including attacks, attack latencies, bites, bouts, pins, flank markings, contact time, and attack types. Attacks were defined as a combination of an approach immediately followed by an attempt to bite (Wommack et al., 2003). Attack latencies were recorded as the duration of time interval between the onset of the task until the resident hamster performed the first attack. Bites were recorded when the residents performed an attack and successfully bit the intruders. Bouts were recorded for each time the resident animal initiated and maintained contact with the intruder for at least 5 seconds (Johnston, 1985). Pins were defined as one animal lying on its back with the other animal on top. Flank markings were identified when hamsters rubbed their flank glands on the walls of cages (Ferris et al., 1988), which were usually performed by residents after successful attacks. Contact time was recorded for the total duration of the testing period when residents maintained physical contact with intruders. Attacks per bout were calculated by dividing the total number of attacks by the total number of bouts. Attack types were recorded as the area on the body of the intruder that the resident initially attempted to bite during an attack. In early puberty, hamsters only target the face (facial attack) or sides of the trunk (lateral attack) of intruders

(Wommack et al., 2003). The percentage of each attack type was calculated by dividing each of the two categories of attacks by the total number of attacks.

Data Analysis

Nonparametric data (behavioral frequencies) were compared between groups through Kruskal-Wallis tests followed by Mann-Whitney tests. Parametric data (duration of time, attacks per bout, and percentages) were compared between groups through one-way ANOVAs followed by Fisher's LSD tests.

Experiment 3: Development of Vasopressin Fiber Density in Early Puberty

Experimental Design

Two groups of male hamsters on P-28 (n=8) and P-35 (n=10) were decapitated. Their brains were removed and placed in 10% Acrolin (Alfa Aesar, Ward Hill, MA) diluted with 0.1M potassium phosphate buffered saline (KPBS, pH=7.2) for 6 hours at room temperature. Then, brains were placed in 20% sucrose in KPBS at 4^0C for at least 24 hours. Then, brains were sliced into 40 μm-thick coronal sections with a freezing rotatory microtome. Brain sections were saved in a cryoprotectant (Watson et al., 1986) at -20^0C until labeled by immunocytochemistry.

Immunocytochemistry

First, brain sections were washed in 0.1M phosphate buffered saline (PBS) to remove the cryoprotectant, and the sections were treated with 1% sodium borohydrate in PBS for 10 minutes to remove the residual aldehydes. After several washes, brain

sections were pre-incubated in 20% NDS, 1% hydrogen peroxide and 0.3% Triton X-100 prepared in a 0.1M PBS. Then, the sections were incubated for 48 hours at 4ºC with a primary antibody (a mouse monoclonal anti-AVP, BER-312, 1/6000, generously donated by Dr. F. Robert, INSERM, Marseilles, France) recognizing the six amino acid cyclic part of vasopressin (Robert et al., 1985, 1991) prepared in "PBS wash" solution (0.1M PBS containing 2% NDS and 0.3% Triton X-100). After several washes, the sections were incubated for 45 minutes at room temperature with a secondary antibody, a biotinylated donkey anti-mouse IgG (9 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) prepared in "PBS wash" solution. After a 45-minute tertiary incubation with an avidin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories, Inc., Burlingame, CA, USA) and several more washes, the sections were labeled with diaminobenzidine (0.05% in 0.1M PBS) to form a brown precipitate over vasopressin cells. The specificity of the AVP antibody was previously tested (Robert et al., 1985) and this procedure was previously validated in hamsters through omission of the primary antibody and preincubation in excess amount of AVP (Delville et al., 2000). The resulting distribution of AVP immunoreactive (AVP-ir) cells and fibers were consistent with previous observations in hamsters using other antibodies or *in situ* hybridization (Delville et al., 1998; Ferris et al., 1995; 1996).

Quantification of Fiber Density

The procedures for quantifying the AVP-ir fibers density were similar to a pervious study (Delville et al., 1998). All slices of brain sections were coded randomly to ensure blind measuring. Images were digitized through a video camera connected with an Apple computer through a frame grabber. The illumination was kept constant for all measurements. A gray-scale normalization was performed to minimize background variations of all images (Delville et al., 2000). AVP-ir fibers density was counted within a circle of 150 μm radius placed on the areas of interest using NIH Image (v. 1.62, NIH, Bethesda) after gray-scale thresholding. Areas selected for quantification included the AH, the posterodorsal part of the medial amygdaloid nucleus (MePD), and the medial preoptic nucleus (MPN), a nucleus with AVP fibers from the suprachiasmatic nucleus (SCN), was quantified as a control. Because AVP neurons in SCN are not associated with agonistic behaviors in hamsters (Delville et al., 1998), the increased AVP-ir fibers in MPN represented the development of the AVP system not specific to aggressive play fighting. For each area, 4-7 consecutive sections were measured bilaterally in each animal (8-14 measures per animal).

Data Analysis

These measures were averaged for each individual, and the averages were compared between groups with independent Student t-tests (two-tailed).

Results

Experiment 1: Vasopressin Neurons with c-Fos-ir labeling

Vasopressin-immunoreactive cells were observed in the hypothalamus within the NC, the PVN, the mSON and the lSON, as previously described (Ferris et al., 1995). The proportion of AVP-ir cells also expressing *c*-Fos-ir was 3 to 4 times higher in experimental animals within the NC [$t(18)=2.68$, $p<0.05$] and the mSON [$t(18)=3.45$, $p<0.01$] (Figs. 3.1 and 3.2). In contrast, the proportion of AVP-ir cells also expressing *c*-Fos-ir did not differ between groups in the PVN [$t(18)=0.475$, $p=0.64$] and the lSON [$t(18)=-0.393$, $p=0.7$].

Experiment 2: Microinjection of Manning Compound into the AH

The data showed that injections of an AVP V1A-receptor antagonist had an inhibitory effect on several aspects of offensive play fighting performed by the residents to the intruders (Fig. 3.5). In particular, the treatment affected attack latencies, attack frequencies, bite frequencies, and the number of attacks per bout. However, no effect was observed on other aspects of agonistic behaviors. Pins were observed in most animals with treatment of different doses. Flank markings were performed in a few animals in three groups at low frequencies. Total numbers of contact bouts and total contact time were not different between groups. In addition, relative proportions of frontal and side attacks were not altered by the treatment.

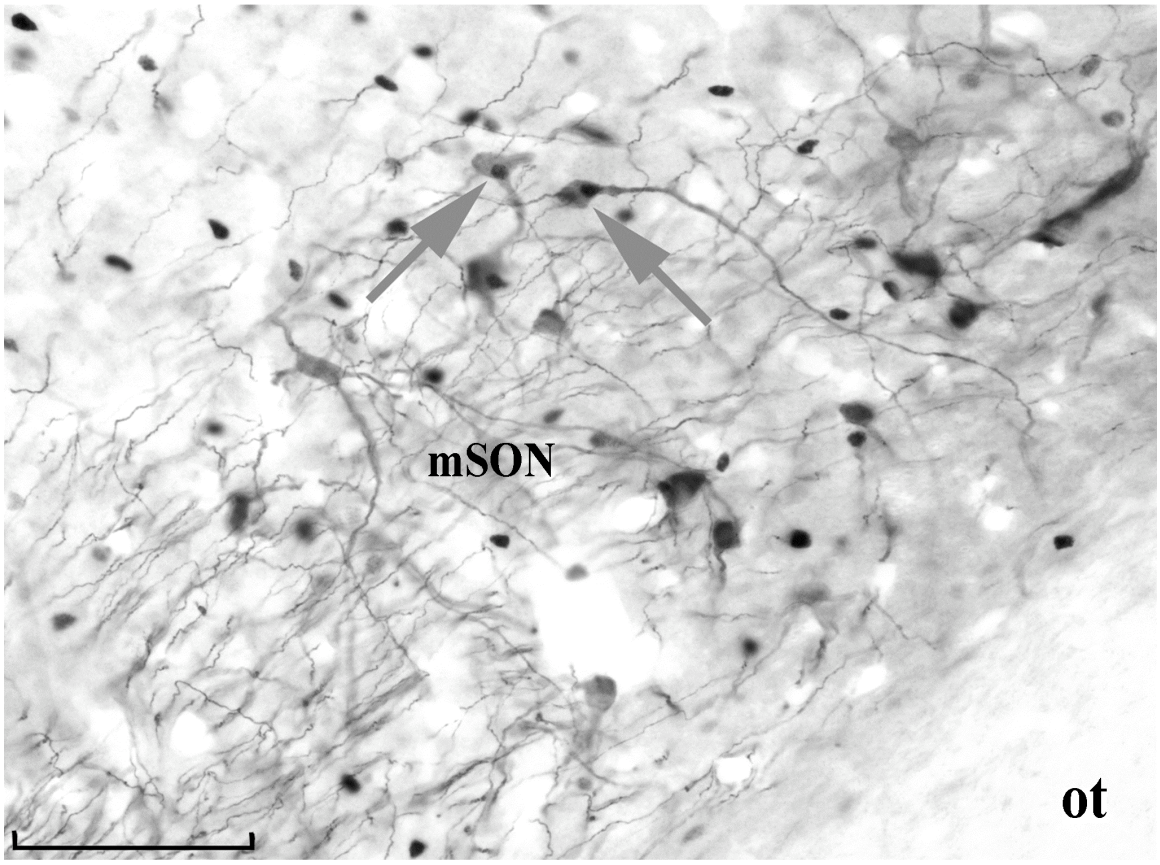
Specifically, injections of Manning compound inhibited attack frequencies at both doses [$H(2)=10.344$, $p<0.01$; 9μM: $U=23$, $p<0.05$; 90μM: $U=10.5$, $p<0.01$]. There was a 75% inhibition of averaged attack frequencies at 90μM and a 50% inhibition at 9μM. Overall, the antagonist increased attack latencies [$F(2,29)=3.418$, $p<0.05$]. While injections with 9μM only had a limited impact ($p=0.078$), the effect was statistically significant with 90μM treatment ($p<0.01$) (Fig. 3.5). Similar observations were made for bite frequencies and the number of attacks per bout [respectively, $H(2)=7.098$, $p<0.05$; $F(2,29)=4.2$, $p<0.05$]. Injections with 90μM Manning compound significantly inhibited both aspects of play fighting [respectively, $U=21$, $p<0.01$; $p<0.01$), while the lower dose had no statistically significant effect (Fig. 3.5).

Treatment with Manning compound outside the AH within the mPOA and the VMH had no statistically significant effects on their play fighting activity.

Experiment 3: Vasopressin Fiber Density

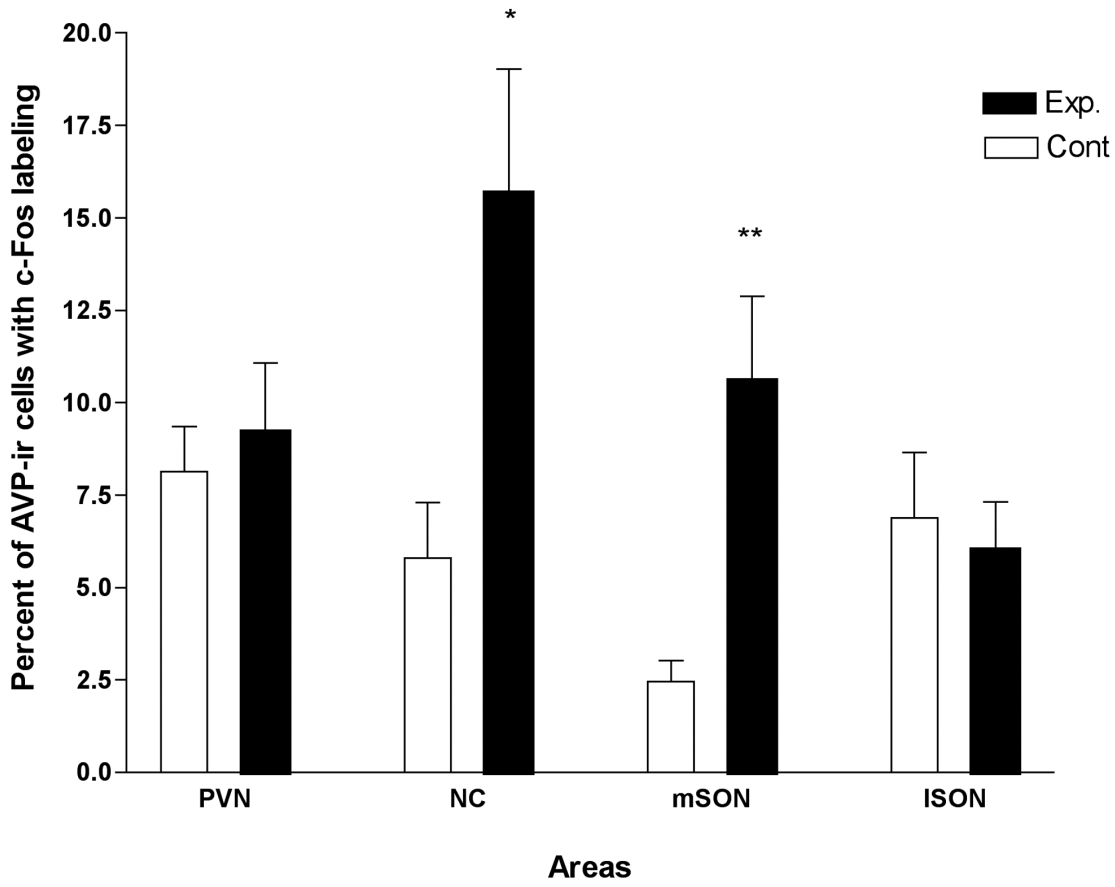
AVP-ir fibers were seen in all selected areas (Fig. 3.6). The density of AVP-ir fibers differed only in one selected area between groups. There was a 90-100% increase in the density of AVP-ir fibers in hamsters from P-28 to P-35 in the MePD [$t(15)=2.92$, $p<0.01$]. There was no difference in the fiber density in other brain areas, including the AH [$t(16)=2.21$, $p=0.052$], and the MPN [$t(16)=0.45$, $p=0.66$] (Fig. 3.7).

Figure 3.1: AVP cells with c-Fos labeling



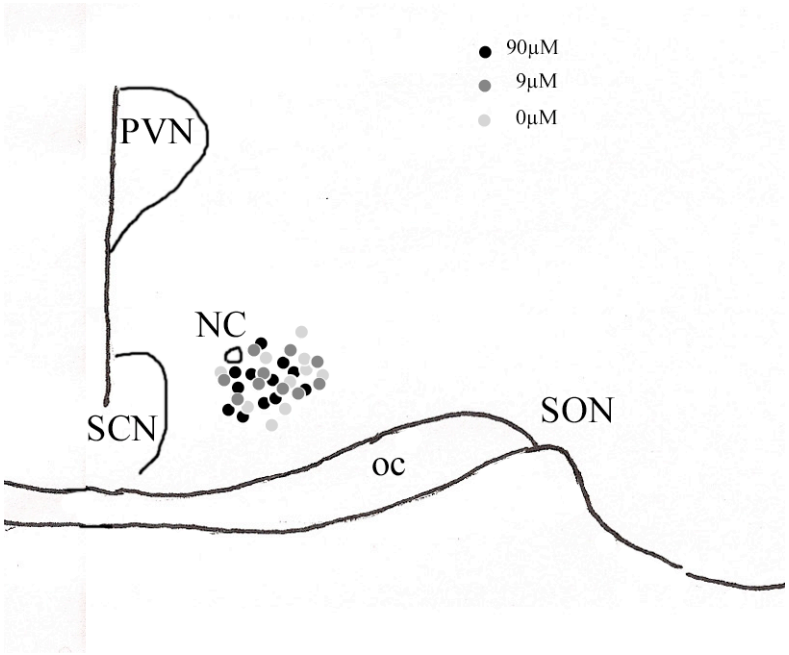
Photomicrograph showing vasopressin neurons in the medial division of the supraoptic nucleus (mSON) also containing *c*-Fos-ir labeling (indicated by arrow heads).
Ot: optic tract. Scale bar = 100μm.

Figure 3.2: AVP and c-Fos double-labeling results



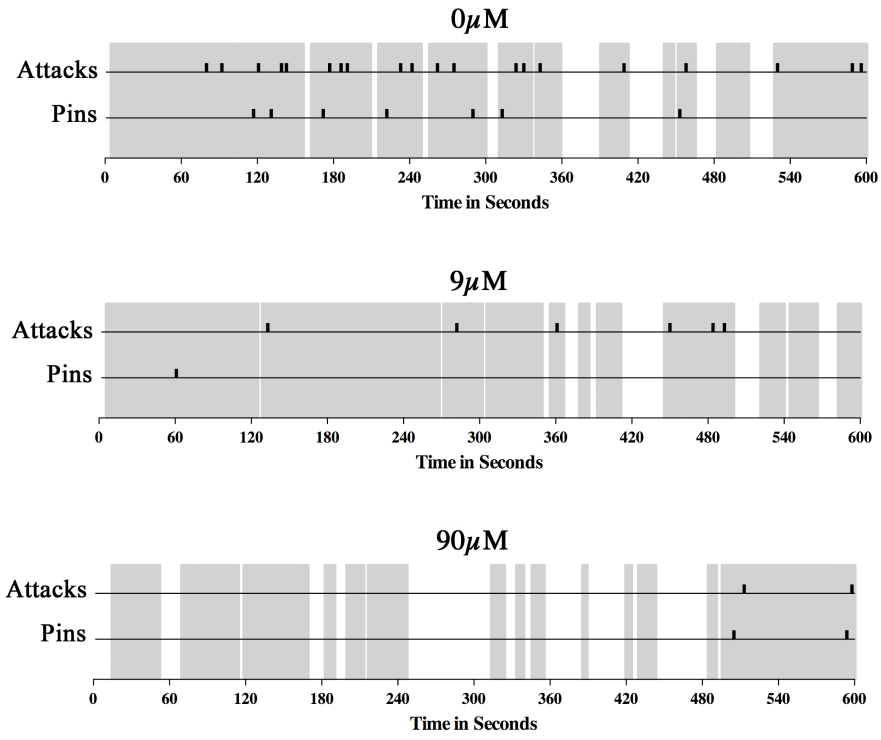
Comparison of the percentages of the vasopressin cells with *c*-Fos-ir labeling between control animals (exposed to a woodblock, n=10) and experimental animals (attacking an intruder, n=10) in the paraventricular thalamic nucleus (PVN), the nucleus circularis (NC), the medial division of the supraoptic nucleus (mSON), and the lateral division of the supraoptic nucleus (lSON).
*= $p<0.05$; **= $p<0.01$ (Student t-test).

Figure 3.3: summary of injection sites within the AH



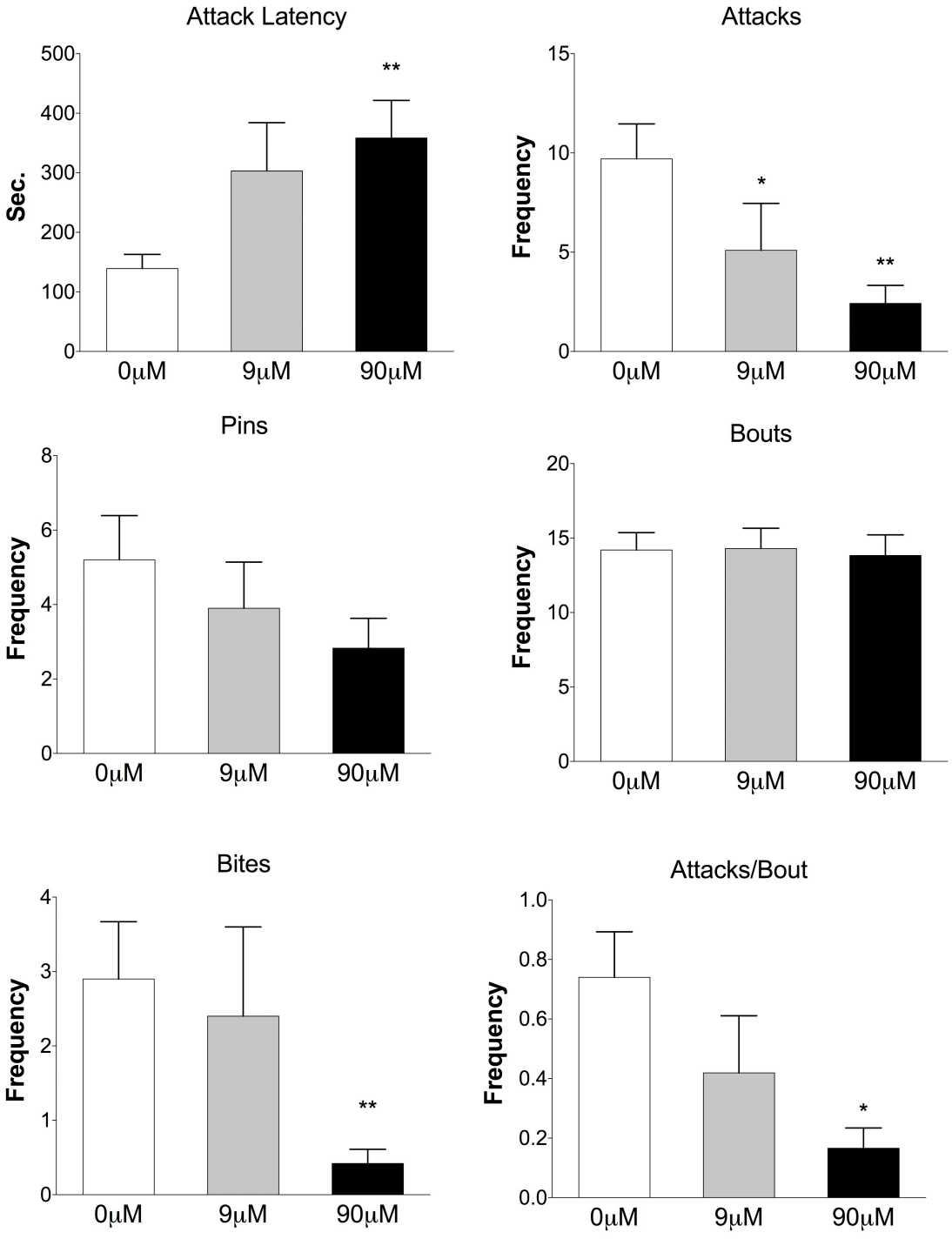
A summary figure showing correct microinjection sites within the AH (light grey circle: 0μM, grey circle: 9μM, and black circle: 90μM).
NC: nucleus circularis; OC: optic chiasm; PVN: paraventricular hypothalamic nucleus; SCN: suprachiasmic nucleus; SON: supraoptic nucleus.

Figure 3.4: examples of temporal distribution of agonistic behaviors



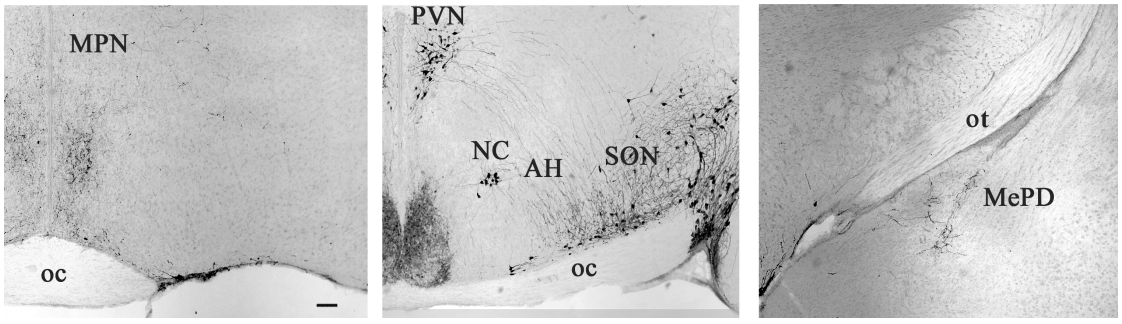
Examples of the temporal distribution of agonistic behaviors (attacks and pins) and the contact time for three representative animals receiving different doses of Manning compounds: 0μM, 9μM, and 90μM. The tests lasted for 10 minutes and the agonistic behaviors were recorded on a second-by-second basis. Each bar represents a single attack or pin. Gray shaded areas indicate the contact time.

Figure 3.5: dose effects of Manning Compound on agonistic behaviors


Attack Latency
Sec.
500
400
300
200
100
0
**
0μM
9μM
90μM
Attacks
Frequency
15
10
5
0
*
**
0μM
9μM
90μM
Pins
Frequency
8
6
4
2
0
0μM
9μM
90μM
Bouts
Frequency
20
15
10
5
0
0μM
9μM
90μM
Bites
Frequency
4
3
2
1
0
**
0μM
9μM
90μM
Attacks/Bout
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
*
0μM
9μM
90μM

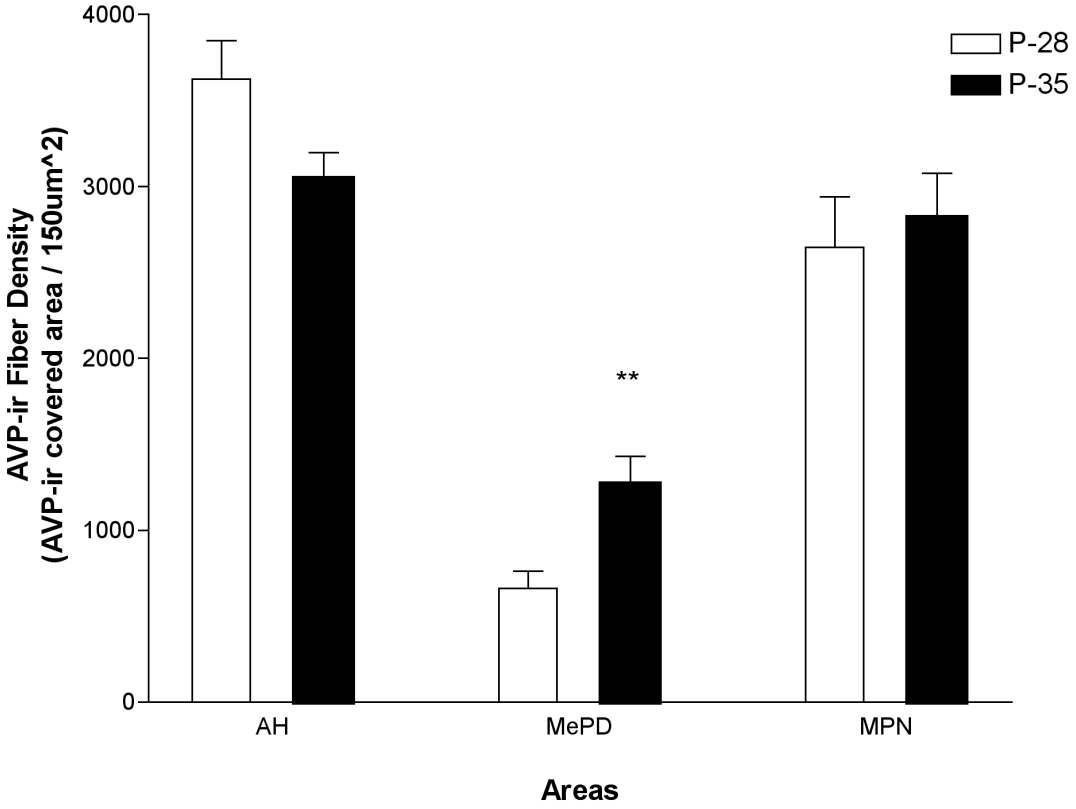
Dose effects of Manning compound on attack latencies, attack frequencies, pin frequencies, bout frequencies, bite frequencies, and the averaged number of attacks per bout of juvenile hamsters during a 10-min resident/intruder task. Error bars denote S.E.M. *= $p<0.05$; **= $p<0.01$ (Numbers of behaviors, including attacks, bites, bouts, and pins, were compared by non-parametric Kruskal-Wallis tests followed by Mann-Whitney tests for comparing between groups. Parametric data, including attack latencies, and the number of attacks per bout, were compared by one-way ANOVAs followed by Post Hoc Tests for between-group comparison).

Figure 3.6: example photomicrographs of AVP-ir fibers



Example photomicrographs showind the distributions of vasopressin fibers. AH: anterior hypothalamus; MePD: the medial amygdaloid nucleus; MPN: the medial preoptic nucleus; NC: nucleus circularis; Oc: optic chiasm; Ot: optic tract; PVN: paraventricular hypothalamic nucleus. Scale bar = 100μm.

Figure 3.7: AVP fiber density between P-28 and P-35



Results of AVP fiber density in selected brain areas in juvenile hamsters between P-28 and P-35. AH: anterior hypothalamus; MePD: the posterior dorsal part of the medial amygdaloid nucleus; MPN: the medial preoptic nucleus.

Discussion

In current study, AVP facilitates the offensive component of play fighting in juvenile hamsters. Neural activity of AVP cells within the NC and the mSON increased in animals performing play fighting. Microinjections of an AVP V1A receptor antagonist into the AH inhibited the intensity of play-fighting attacks. In addition, the increased frequency of play fighting performed by juvenile hamsters in the first week of early puberty was correlated with the enhanced density of AVP fibers in one limbic area associated with play fighting. These results further support the similarities in neural mechanisms between serious play fighting and offensive aggression in hamsters discussed in Chapter 2.

In adult hamsters, AVP cells in the NC and the mSON are possible sources of vasopressinergic innervations to the AH for controlling agonistic behaviors in hamsters (Ferris et al., 1990; Mahoney et al., 1990). In adult hamsters, *c*-Fos expression was enhanced within the NC and the mSON after performance of offensive aggression (Delville, et al., 2000). These results indicate that the AH is one of the primary target areas for AVP action in controlling aggression. In the first experiment, AVP cells within the same areas showed 3 to 4 times increased expression of *c*-Fos-ir labeling in animals after performing offensive play fighting. This result is consistent with the previous findings in adults and further supports my hypothesis of a common neural circuitry controlling the offensive components of both serious play fighting and aggression. In addition, this finding also indicates that the enhanced activity of AVP

neurons in the AH may be a central element of the core neural circuitry controlling both play fighting and aggression. This possibility was tested in the second experiment.

In the second experiment, blockade of AVP V1A receptors within the AH inhibited the offensive component of play fighting (i.e., attacks, bites) in juvenile hamsters. In addition, injections located outside the AH had no effect on the behaviors. These data support the hypothesis that the offensive component of play fighting is controlled by AVP release within the AH. These data are consistent with the enhanced activity of AVP neurons after consummation of play fighting in the first experiment, even though the activation is not as intense as reported for adults (Delville et al., 2000). As offensive responses by juvenile hamsters mature gradually from play fighting into aggression during puberty, our data suggest that AVP modulate these responses throughout their development. The present data are the first to show a role for AVP and the AH in the control of play fighting.

The inhibitory effect of Manning compound on play fighting was specific to aspects of the attack components of this behavior, including attacks, bites, and attack latencies. Since the treatments did not affect bouts and contact time, these results indicate that the inhibitory effects of Manning compound were not the result of a non-specific behavioral inhibition. Manning compound did not inhibit flank marking in juvenile hamsters as reported in adults (Ferris et al., 1988). The reason could be the low frequency of flank marking performed at this age. The treatment decreased the

frequency of pins, although the effect was not statistically significant. During play fighting, pins may represent a different, possibly more playful, aspect of this behavior (Pellis and Pellis, 1991; Reinhart et al., 2004; Siviy et al., 2003). Even though we could not eliminate the possibility that AVP has an effect on pins in play fighting, based on my experimental results, it is likely that pins would be mediated through another neural substrate. Together, these data suggest that AVP in the AH controls the onset of offensive responses, such as attacks, whether during play fighting or adult aggression. Though the attack types are different between these two behaviors, AVP controls the activation of the behavior, and it is likely that other neural systems connected to the AH determine how the motor response is performed. The lack of effect of treatment on attack types also supports this idea.

In juvenile hamsters, play fighting is composed of more repetitive attacks compared with adult animals (Wommack et al., 2003; Taravosh-Lahn and Delville, 2004). My finding shows that a vasopressin V1A-receptor antagonist in the AH significantly decreased the number of attacks per bout. Since the treatment had no effect on the number of bouts, the result could be explained as the treatment significantly decreasing the repetitive play fighting attacks in juvenile hamsters. These effects of Manning compound on attack repetitions suggest that it is one of the mechanisms of action of AVP at least in juvenile hamsters. Interestingly, serotonin (5-HT) also seems to affect play fighting through similar mechanism (Taravosh-Lahn et al., 2006). Peripheral injections of a high dose of a serotonin reuptake inhibitor

decreased play fighting in juvenile hamsters particularly in the number of attacks per bout. In adult hamsters, the neural circuitry controlling offensive aggression, modulated by AVP and 5-HT in the AH, has been well studied (Ferris and Delville, 1994; Ferris et al., 1997). Both findings in juvenile hamsters indicate that the AH neural circuitry, mediated by AVP and 5-HT, has been established before puberty and facilitates aggression through the development in puberty in hamsters. Specifically, the role of AVP in the AH is to turn on or off the offensive components of agonistic behavior in hamsters.

The results of the development of AVP-ir fiber density are unexpected. The results of the first two experiments show that AVP in the AH facilitates play fighting. However, I could not find an increased density in the AVP-ir fiber in the AH in hamsters between P-28 and P-35 when the frequency of this behavior performed in juveniles significantly increases. This result could be explained as AVP in the AH is not responsible for the development of this agonistic behavior. Interestingly, an enhanced density of AVP-ir fibers was found in the MePD, another important brain area regulating agonistic behaviors. Based on these findings, it is possible that AVP outside of the AH is responsible for the development of play fighting.

In conclusion, these findings indicate that AVP released into the AH specifically controls the onset and frequency of offensive responses, such as attacks, in juvenile hamsters. It is possible that other aspects of play fighting, such as pins, are

controlled by different neurotransmitter systems. This possibility is discussed in the next chapter.

Chapter 4: CRH and Play Fighting

Introduction

In last chapter, my results show a very selective effect of AVP and the AH on play fighting. Since play fighting is a complex social behavior including different aspects, it is possible that other brain areas and other neurotransmitter systems may have more a more general role on mediating other aspects of this behavior. I predict that, in hamsters, neural systems known to have more general effects on adult aggression could also have more general effects on play fighting. In adult hamsters, blockade of CRH receptors inhibits aggression (Farrokhi et al., 2004). In that study, the inhibitory effect of treatment also includes contact time, suggesting a motivational role for this neuropeptide, though the site of action remains unclear. CRH is associated with aggression in rats as well. Infusions of CRH intracerebroventricularly (i.c.v.) or into the amygdala facilitate aggressive behavior in rats (Tazi et al., 1987; Elkabir et al., 1990).

Because CRH has a broad effect on facilitating aggression in adult hamsters, I predicted that CRH also plays a general role in facilitating play fighting. Also, if CRH enhances play fighting, then it is possible that this peptide is associated with the activation of play fighting in early puberty. In this study, I looked for neural sites showing enhanced CRH immuno-reactive (CRH-ir) fiber innervations in early puberty as potential sites of action for CRH in play fighting. Then, I injected a CRH

antagonist in these areas to confirm the role of this neuropeptide in the control of this behavior. Finally, I also attempted to determine the possible origin of CRH neurons involved in play fighting through double labeling procedures with a marker of neuronal activity.

General Method: Animals and Treatment

The present studies were carried out with male golden hamsters (*Mesocricetus auratus*) bred in the laboratory from a colony originating from Harlan Sprague Dawley (Indianapolis, IN, USA). All litters were culled to six pups (4 males, 2 females) by Postnatal days 7 (P-7). All males were weaned on P-25 and housed individually in plexiglass cages (20 x 33 x 13 cm). The hamsters were housed under a reversed light cycle (14:10 light/dark cycle and lights off at 10:00 h) and received food and water *ad libitum*. Their body weights were measured weekly and recorded to monitor their development. The studies were conducted in early puberty (between P-28 to P-35) around the time of peak play fighting activity in this species (Goldman and Swanson, 1975; Taravosh-Lahn and Delville, 2004; Cervantes *et al*., 2006). All procedures were performed according to National Institutes of Health guidelines approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin and conducted in an AALAC-accredited facility.

Methods of Experiment 1: CRH Fibers Density

Experimental Design

Two groups of male golden hamsters, P-28 (n=8) and P-35 (n=9), were sacrificed for CRH-ir fiber staining and quantification in selected limbic areas. The procedure was the same as been described in the Experiment 3 in Chapter 3.

Immunocytochemistry

The procedure for CRH immuno-staining performed was similar as the procedure for AVP immuno-staining described in the Experiment 3 in Chapter 3, except that a 0.05M Tris-buffered saline (TBS) was used as a buffer solution in all procedures, a rabbit polyclonal antibody to Human/Rat CRH (dilution 1:6000; Peninsula Laboratories, Inc., San Carlos, CA, USA; Cat. No. T-4037), prepared in "TBS wash" solution (0.05M TBS containing 2% NGS and 0.3% Triton X-100) was used as a primary antibody, and a biotinylated goat anti-rabbit IgG (9 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; Lot: T-0411) was used for the secondary antibody.

Quantification of CRH Fiber Density

The procedures for quantifying the CRH-ir fibers density were the same as described in the Experiment 3 in Chapter 3. Areas selected for quantification included the anterior hypothalamus (AH), the lateral septum (LS), the posterior dorsal part of the medial amygdala (MePD), and the ventrolateral hypothalamus (VLH). For each area, 4-7 consecutive sections were measured bilaterally in each animal (8-14 measures per animal).

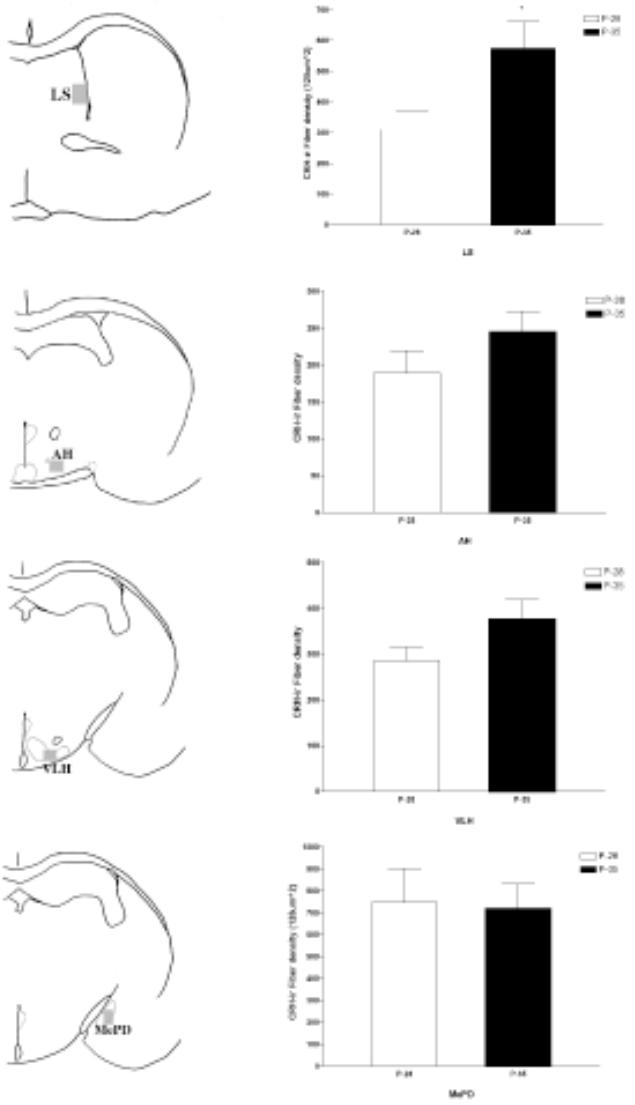
Data Analysis

These measures were averaged for each individual, and the averages were compared between groups with independent Student t-tests (two-tailed).

Results of Experiment 1: CRH Fibers Density

The distributions of CRH immunoreactive (CRH-ir) fibers were similar as been described in adult hamsters (Delville et al., 1992) and fibers were visible in all areas selected for quantification. The density of CRH immunoreactive fibers differed only in one selected area between groups (Fig. 4.1). There was a 90-100% increase in the density of CRH-ir fibers in hamsters from P-28 to P-35 in the LS, $t(15)=2.44$, $p<0.05$. There was no difference in the fiber density in other brain areas, including the AH, $t(15)=1.31$, $p=0.21$, the VLH, $t(15)=1.72$, $p=0.11$, and the MePD, $t(15)=0.159$, $p=0.88$.

Figure 4.1: CRH fiber density between P-28 and P-35



Results of CRH fiber density in selected brain areas in juvenile hamsters between P-28 and P-35. AH: anterior hypothalamus; LS: the lateral septum; MePD: the medial amygdaloid nucleus; VLH: the ventrolateral hypothalamus.

Methods of Experiment 2: Microinjection of CRH antagonist into the LS

Experimental Design

The data from experiment 1 suggested that the LS could be an important area associated with play fighting. In the second experiment, a CRH receptor antagonist was microinjected into this brain area to test play-fighting behaviors.

On P-28, male hamsters were pre-tested for agonistic behavior for 10 minutes individually in the presence of a smaller (10-20% lighter) and younger unfamiliar male intruder. This resident/intruder procedure favors offensive responses by residents (Delville et al., 2003). Animals performing no attack on intruders were excluded from the current study, which usually occurs one in every fifteen animals. Then, animals were divided into three homogeneous groups based on their body weight and the agonistic behaviors observed in this test.

On P-30, hamsters were anesthetized with sodium pentobarbital (90mg/kg, i.p.) and placed in a stereotaxic apparatus. A twenty-six gauge guide cannula was implanted aimed 2mm above the LS. The coordinate of the LS for hamsters at P-30 was 2.2mm anterior to bregma, 0.9mm lateral to midline, and 2.5mm down from dura. The incisor bar was leveled at +1.5mm. After 5 days of rest, microinjections were made through the guide cannula with a 33-gauge needle, cut to extend 2mm beyond the guide cannula tip, attached to a 1μl Hamilton syringe by PE 20 tubing. The animals were injected with either 0ng, 30ng, or 300ng of a CRH antagonist (α-helical CRH, Sigma-Aldrich, Cat No. C2917) dissolved in 100nl of saline. The dosage used

in this experiment was based on a previous study of stress-induced behavior in the LS in rodents (Bakshi et al., 2002). Microinjections were performed without anesthesia. The entire procedure took around 1 minute (30 seconds for drug injection and another 30 seconds for drug absorption with needles staying in the guide cannula). One hour later, animals were observed for agonistic behaviors during a 10-min encounter with another unfamiliar intruder. Agonistic encounters of animals were videotaped with a digital video camera (Sony Digital 8 Handycam, Sony Corporation of America, New York, NY, USA) for later review. Immediately after the resident/intruder test, animals were placed in a Lat Maze for 10 minutes to test their local motor activity. The size of the Lat Maze was the same as described in previous experiments (Taravosh-Lahn et al, 2006; Cervantes et al., 2005). In the maze, hamsters were allowed for free moving in the corridor with lines drawn. The total number of lines crossed was counted. After testing, all animals were sacrificed and their brains were fixed by immersion in 10% Acrolein and then sliced into 40 μm-thick coronal sections with a freezing rotatory microtome. A Nissl stain was performed on these tissues to localize the position of the tip of the 33-gauge microinjection needle. Twenty-four animals with correct injection sites in the LS were included in behavioral analysis (0ng: n=8; 30ng: n=8; 300ng: n=8). Another group of animals receiving microinjections of 30ng of α-helical CRH into the lateral ventricle (n=12) were used for comparison. The coordinate of the

lateral ventricle for hamsters at P-30 was 2.2mm anterior to bregma, 1.1mm lateral to midline, and 2.5mm down from dura.

Behavioral Observations

Agonistic interactions were reviewed with iMovie (Apple Computers, Inc., Cupertino, CA, USA) and quantified with EventMonitor software (courtesy of Dr. J. Alberts, Bloomington, IN, USA) for second-by-second analysis. Agonistic behaviors quantified in this experiment and their definitions were the same as described in the Experiment 2 in Chapter 3, including attacks, attack latencies, bites, bouts, pins, flank markings, contact time, attack per bout, and attack types.

Data Analysis

Nonparametric data (behavioral frequencies and number of lines) were compared between groups through Kruskal-Wallis tests followed by Mann-Whitney tests. Parametric data (duration of time, attacks per bout, and percentages) were compared between groups through one-way ANOVAs followed by Fisher's LSD tests.

Results of Experiment 2: Microinjection of CRH antagonist into the LS

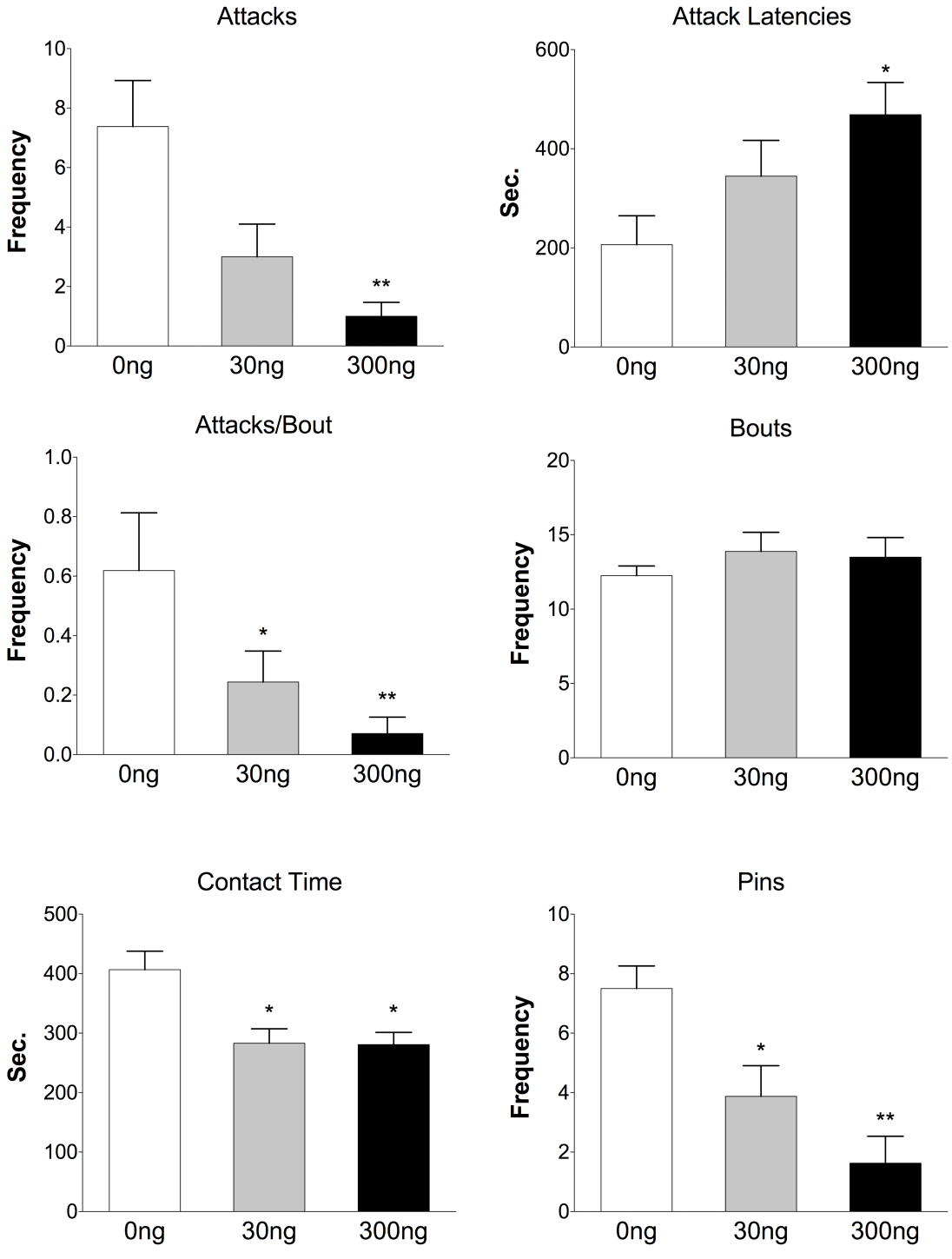
The data showed that injections of a CRH antagonist had an inhibitory effect on several aspects of offensive play fighting performed by the residents to the intruders (Fig. 4.2). In particular, the treatment affected attack latencies, attacks, pins, averaged number of attacks per bout, and total contact time. However, no effect was observed on other aspects of agonistic behaviors. Bites and flank markings were

observed in some animals in all groups at low frequencies. Total numbers of contact bouts were not different between groups. Relative proportions of frontal and side attacks were not altered by the treatment. In addition, there was no difference in the total lines crossed in the Lat Maze test.

Specifically, injections of α-helical CRH inhibited attacks. There was a trend of inhibition at 30ng and a significant inhibitory effect at 300ng [$H(2)=9.584$, $p<0.01$; 30ng: $U=14$, $p=0.065$; 300ng: $U=5$, $p<0.01$]. There was an 85% inhibition of averaged attack frequencies at 300ng and a 50% inhibition at 30ng. Overall, the antagonist increased attack latencies [$F(2,21)=4.017$, $p<0.05$]. The effect only showed up in 300ng treatment ($p<0.05$) but not in 30ng treatment ($p=0.15$). There was a 2-times increase in attack latency at 300ng. The treatment significantly decreased averaged attacks per bout [$F(2,21)=7.635$, $p<0.01$; 30ng: $p<0.05$; 300ng: $p<0.01$]. There was a 90% inhibition of averaged attacks per bout at 300ng and a 60% inhibition at 30ng. Alpha-helical CRH significantly inhibited pins at both dosages [$H(2)=11.94$, $p<0.01$; 30ng: $U=10.5$, $p<0.05$; 300ng: $U=3.5$, $p<0.01$]. There was a 75% inhibition of averaged pins frequencies at 300ng and a 50% inhibition at 30ng. Alpha-helical CRH significantly decreased total contact time at both dosages [$F(2,21)=7.926$, $p<0.01$; 30ng: $p<0.01$; 300ng: $p<0.01$]. There was a 30% decrease in total contact time with treatment at both dosages.

Treatment with α-helical CRH into the lateral ventricle had no statistically significant effect on play-fighting activity compared with animals treated with saline into the LS.

Figure 4.2: results of alpha-helical CRH on agonistic behaviors


Attacks
Frequency
10
8
6
4
2
0
**
0ng
30ng
300ng
Attack Latencies
Sec.
600
400
200
0
*
0ng
30ng
300ng
Attacks/Bout
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
*
**
0ng
30ng
300ng
Bouts
Frequency
20
15
10
5
0
0ng
30ng
300ng
Contact Time
Sec.
500
400
300
200
100
0
*
*
0ng
30ng
300ng
Pins
Frequency
10
8
6
4
2
0
*
**
0ng
30ng
300ng

Dose effects of alpha-helical CRH on attacks, attack latencies, averaged number of attacks per bout, bouts, pins, and total contact time on juvenile hamsters during a 10-min resident/intruder task. Error bars denote S.E.M. *= $p<0.05$; **= $p<0.01$ (Numbers of behaviors, including attacks, bouts, and pins, were compared by non-parametric Kruskal-Wallis tests followed by Mann-Whitney tests for comparing between groups. Parametric data, including attack latencies, averaged number of attacks per bout, and total contact time were compared by one-way ANOVAs followed by Post Hoc Tests for between-group comparison).

Methods of Experiment 3: Immunocytochemistry to c-Fos Combined With CRH

Experimental Design

The experimental procedure by using the resident/intruder task was the same as been described in Chapter 2. There were total 23 animals on P-35 included in this experiment (n=11 in the control group, n=12 in the experimental group). The procedure for perfusion was the same as described in Chapter 2 except that a different fixative solution containing 4% paraformaldehyde in 0.1M potassium phosphate buffered saline (KPBS, pH 7.4) was used and brains were put in the same fixative solution for a 30-minute post fixation after been removed from the skulls.

Combined Immunocytochemistry

First, Procedures for immuno-labeling *c*-Fos and CRH were similar as the procedures for immuno-labeling *c*-Fos and AVP described in the Experiment 1 in Chapter 3. For c-Fos labeling, a goat polyclonal primary antibody to *c*-Fo*s* (0.05μg/ml, sc-52-G, Lot L1406, Santa Cruz Biotechnology Inc., Santa Cruz, CA) recognizing an sequence (residues 1-16) at the N-terminus of *c*-Fos of human origin (Finkel et al., 1966; Nishizawa et al., 1987) and a secondary antibody (biotinylated donkey anti-goat IgG, 2.5 μg/ml, Lot 81450, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) prepared in "KPBS wash" solution (0.05M KPBS with the presence of 2% NGS and 0.3% Triton X-100) were used. For CRH labeling, a primary antibody (a rabbit anti-CRF, 1/1000, Cat. No. T-4037, Peninsula Laboratories,

LLC, San Carlos, CA) and a secondary antibody (a biotinylated donkey anti-rabbit IgG, 9 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) prepared in "TBS wash" solution (0.05M TBS containing 2% NDS and 0.3% Triton X-100) were used. After labeling, double-labeled cells can be visualized with a brown soma for CRH and a black nucleus for *c*-Fos expression.

Quantification of Immunocytochemistry

The proportions of CRH-ir cells also containing *c*-Fos-ir were counted from camera lucida drawings taken consecutive sections of the brains. In hamsters, the locations of CRH neurons are similar to the distributions in rats (Delville et al., 1992). The CRH neurons are mainly located in the central amygdala (CeA), the BST, the PVN, and several limbic areas. CRH neurons in the CeA and the BST are too densely packed to be quantified. The areas selected for quantification include the prefrontal cortex (PFC), the Diagonal Band of Broca (DBB), the preoptic area (POA), the reuniens thalamic uncleus (Re), and the PVN. In these areas, double-labeled cells were analyzed as the percentage of CRH-ir cells also containing *c*-Fos-ir labeling. Six to ten measures were taken from each individual in each area. These measures were averaged for each individual and compared between groups.

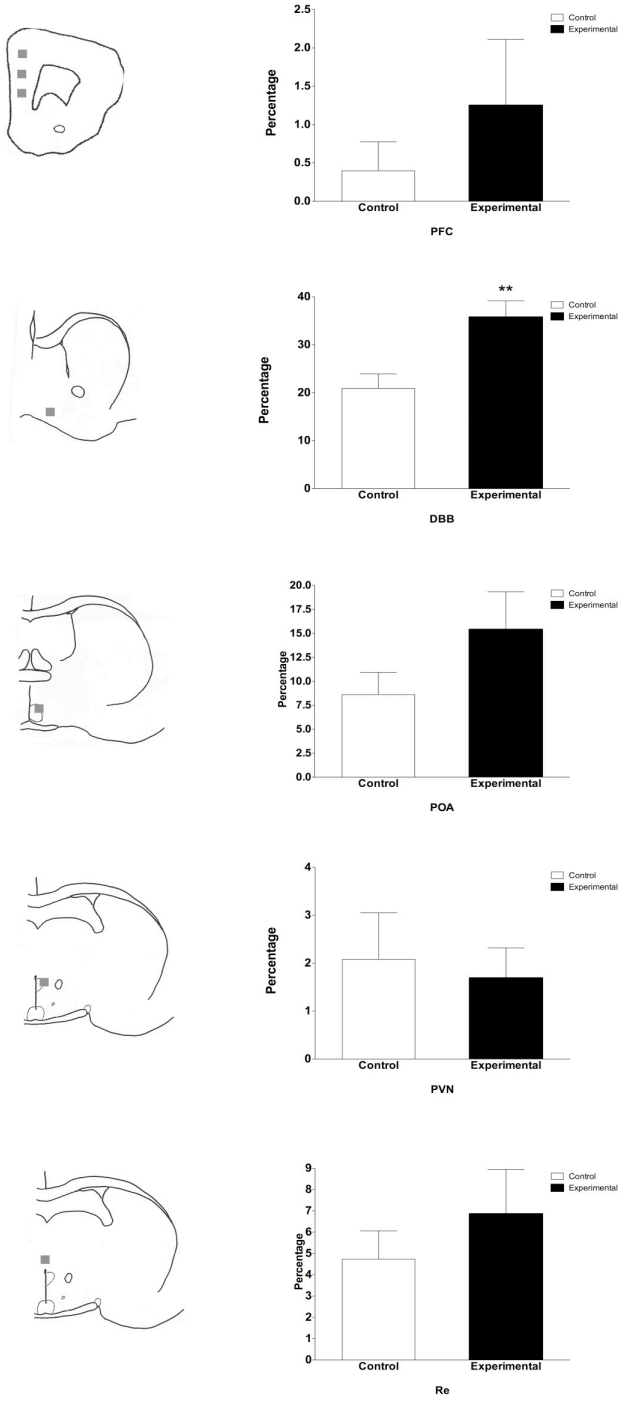
Data Analysis

All anatomical measures were compared between groups with independent Student t-tests (two-tailed) for each area sampled.

Results of Experiment 3: Immunocytochemistry to c-Fos Combined With CRH

CRH-immunoreactive (CRH-ir) cells were observed mainly in the CeA, the BST, the PVN, and other limbic areas as previously described in hamsters and rats (Delville et al., 1992; Swanson et al., 1983). In all selected areas, there was only one area with CRH neurons showing significantly enhanced *c*-Fos-ir expression (Fig. 4.3). The proportion of CRH-ir cells also expressing *c*-Fos-ir was 2 times higher in experimental animals within the DBB [$t(21)=3.28$, $p<0.01$]. In contrast, the proportion of CRH-ir cells also expressing *c*-Fos-ir did not differ between groups in the PVN [$t(21)=0.346$, $p=0.73$], the POA [$t(20)=1.49$, $p=0.15$], the Re [$t(21)=0.846$, $p=0.41$], and the PFC [$t(18)=0.873$, $p=0.39$].

Figure 4.3: c-Fos and CRH double-labeling results



Comparison of the percentages of the CRH cells with *c*-Fos-ir labeling between control animals (exposed to a woodblock) and experimental animals (attacking an intruder) in the the prefrontal cortex (PFC), the Diagonal Band of Broca (DBB), the preoptic area (POA), the reuniens thalamic uncleus (Re), and the paraventricular thalamic nucleus (PVN).
*= $p<0.05$; **= $p<0.01$ (Student t-test).

Discussion

Based on previous studies with adult hamsters, I predicted a role for CRH on play fighting. In particular, a broader role than for AVP was predicted. Furthermore, I also predicted a correlation between the increased play fighting activity in early puberty and CRH availability in specific bran areas responsive to the neuropeptide. Indeed, in the first study, I found increased CRH innervations during early puberty particularly within the LS. Second, microinjections of a CRH receptor antagonist within this area inhibited several aspects of play fighting including attacks, attack latencies, averaged attacks per bout, pins, and total contact time. Thus, CRH plays a broader role than AVP on play fighting. Finally, I identified a brain area, DBB, as a possible source for CRH innervations of the LS.

The behavioral effect of the treatment of a CRH antagonist observed in current study is very interesting. The effects on play fighting are more general than those observed with an AVP receptor antagonist discussed in Chapter 3. It is unlikely that these effects result from a non-specific behavioral inhibition. In this study, animals were observed in a LAT maze to assess locomotor activity. Injections of the CRH antagonist had no effect on this behavior. Thus, the effects observed are selective to play fighting behavior. In addition, the effects are also selective to the LS as injections located just outside the area into the lateral ventricles failed to have any inhibitory effects.

The focus of this study on the LS is also very suggestive. In Chapter 2, I note a large activation of c-Fos immunoreactivity within this area in association with the consummaton of play fighting. Such a correlation has never been reported in adults (Delville et al., 2000), and may be specific to younger animals. It is possible, though not necessarily, that CRH in the LS plays a unique role in hamsters. It should be noted that the LS and the AH are known to have extensive reciprocal connections in rodents, including this species (Ferris et al., 1990b). In previous studies, the LS was hypothesized to be a control area located ahead of the AH in the control of flank marking, another form of agonistic behavior, in this species (Ferris et al., 1990b, 1993, 1994; Irvin et al., 1990). It was hypothesized, then, that the area integrates an olfactory and a memory input necessary to the formation of olfactory memory and necessary to the decision of whether an animal should flank mark or fight in the presence of an intruder (Ferris et al., 1993). A related role may be possible in juveniles. An area integrating olfactory with memory input could also play a key role in play fighting. Since CRH in the area has a more general role, then it could be argued that the LS determines the onset of play fighting activity not specifically attacks, then passes the hand to the AH for attack initiations.

In this study, I also found CRH neurons in the DBB showing enhanced activity in association with play fighting. This area is highly interconnected with the LS and ventral striatum in rats (Jakab and Leranth, 1995), although this connection has not been described in hamsters. It is possible that these CRH neurons in the DBB would

be part of a motivational component activating play fighting in juvenile hamsters through providing direct anatomical inputs to the LS. Further studies are necessary to confirm this possibility.

In conclusion, CRH in the LS play an important role in regulating play fighting in juvenile hamsters. The DBB may provide the source of CRH cells controlling this behavior. Compared with the effect of AVP on play fighting, CRH provides a more general control on regulating both aspects of play fighting, pins and attacks.

Chapter 5: General Discussion

Play fighting, a form of agonistic behavior, is commonly observed in juvenile animals (Bekoff and Byers, 1998; Vanderschuren et al., 1997; Blanchard et al., 2003; Delville et al., 2005; Pellis, 2002). On different species, this behavior could be "playful" or "serious" (Pellis and Pellis, 1998; Pellis, 2002). "Playful" play fighting has been well documented in rats and appears rewarding (Pellis, 1988; Panksepp and Burgdorf, 2003). "Serious" play fighting has been observed in hamsters (Goldman and Swanson, 1975; Wommack et al, 2003). In this territorial animal, "serious" play fighting is used to establish dominant/subordinate hierarchies (Delville et al., 2005). Similar to adult aggression, play fighting includes offensive and defensive components (Pellis, 2002; Delville et al., 2005). The neural mechanism controlling play fighting remains poorly understood. In current study, the neural control of the offensive component of "serious" play fighting in juvenile hamsters has been identified.

Neural Circuitry of Play Fighting

The neural circuitry of offensive play fighting in juvenile hamsters has been identified in current study. Brain areas, including the LS, the VLH, the MePD, and the BST, showed enhanced c-Fos expression in juvenile hamsters after the consummation of offensive play fighting. The activated pattern of brain areas in play fighting in juvenile hamsters is similar to that of adult aggression in adult hamsters

(Delville et al., 2000). An enhanced c-Fos expression was also found in the prefrontal cortex (PFC), including the PrL, the IL, and the Cg1, which indicates the involvement of the PFC in play fighting. The PFC may play an inhibitory role in regulating play fighting since play fighting is associated with a de-synchronization of c-Fos expression between the prefrontal cortex and the AH.

C-Fos protein is synthesized from an activation of an immediate early gene and functions as a transcriptional regulator for several genes (Finkel et al., 1966; Nishizawa et al., 1987). C-Fos or other immediate early genes have been used as a marker to identify activated brain areas associated with different kinds of social behaviors for decades (Potegal et al., 1996; Delville et al., 2000; Haller et al., 2006). However, there are some limitations by using c-Fos to study neural networks of social behaviors. C-Fos provides a functional map of brain areas associated with a behavior, but it dose not indicate an anatomical connections between brain areas show enhanced c-Fos activity. Neurons with c-Fos activity are not necessarily inter-connected, either. Also, brain areas with no enhanced c-Fos activity should not be interpreted as these brain areas are not associated with the behavior. For example, there was no statistically significant enhanced c-Fos activity in the AH in hamsters after the consummation of play fighting (discussed in chapter 2). However, the AH turns out to be an important area for AVP to facilitate play fighting (discussed in chapter 3). Other methodologies, like microinjections, could provide information of brain areas, not been reported to be important in the c-Fos study, in regulating play fighting.

I suggest a play fighting neural circuitry, which includes the AH, the LS, the BST, the MePD, the VLH, and the PFC, modulating offensive play fighting in juvenile hamsters (Fig. 5.1). Several brain areas in this circuitry, including the BST, the AH, the LS, and the MePD, overlap with brain areas belonging to a social behavior neural network modulating several kinds of social behaviors, including mating, parental behavior, and agonistic behavior in mammals (Newman, 1999). Further studies indicate that this social behavior neural network also exists in other vertebrates, including birds, fishes, and reptiles (Goodson, 2005; Crews, 2003). It is suggested by Newman that for mammals, the neural circuitry contains six brain areas, including the AH, the MeA/BST, the LS, the POA, the ventromedial hypothalamus (VMH), and the midbrain. These six brain areas are heavily interconnected and all areas contain sex hormone receptors susceptible for hormonal changes during sexual maturation (Newman, 1999). Different social behaviors are controlled by different activation patterns in the same neural circuitry. Furthermore, all nodes in this circuitry are sexual-steroid sensitive. Therefore, this circuitry is dynamically modulated through life by sexual maturation, by experience, by learning, by sensory stimulations, and by reproductive cycles (Newman, 1999). However, all reports about the social behavior neural network are based on experimental data from adult animals. Little is known about how this circuitry works in juveniles. My finding shows that there is a large overlap in brain areas between the play fighting neural circuitry and the social behavioral neural circuitry. This result indicates that the social behavioral neural

circuitry is established ahead of puberty, and controls agonistic behavior throughout the entire peri-pubertal period from play fighting to aggression. Furthermore, in the following two sections, I am going to discuss how different nodes in the ply fighting neural circuitry, the AH and the LS, control different aspects of play fighting.

Vasopressin and Play Fighting

Vasopressin plays a key role in the control of offensive aggression in adult male hamsters (Ferris and Delville, 1994; Ferris et al., 1997). In my study, AVP facilitates play fighting. Microinjections of an AVP V1A receptor antagonist into the AH inhibits play fighting. The results of c-Fos and AVP double labelings indicate that the NC and the mSON are two possible source areas of AVP neurons involved in regulating this behavior. A similar finding has been reported in adult hamsters under similar test condition (Delville et al., 2000).

Vasopressin in the AH controls very specifically the attack of play fighting. The inhibitory effects of Manning compound on play fighting are specific to some components of this behavior, including attacks, bites, and attack latencies, but not to other aspects of play fighting, like pins and total contact time. The role of AVP in the AH in controlling play fighting is the same as been reported in regulating aggression in adult hamsters (Ferris et al., 1997; Ferris and Potegal, 1988). These data not only support the idea that a common social behavior neural circuitry controls agonistic behaviors in hamsters through development, but also describe how a neurotransmitter

system (AVP) facilitates agonistic behaviors in one important brain area (the AH) of the social behavior neural circuitry. My findings suggest that AVP in the AH controls the onset of offensive responses, such as attacks, through development from play fighting to adult aggression. Though the attack types are different between these two behaviors, AVP in the AH specifically controls the activation of attacks. The different attack types between juvenile and adult hamsters are possibly due to the hormonal changes during puberty.

CRH and Play Fighting

Vasopressin in the AH has a very selective effect on play fighting. It is possible that other brain areas in the social behavior neural circuitry and other neurotransmitter systems have a more general control of other aspects of this agonistic behavior. This possibility is tested in the CRH system.

In my study, microinjections of a nonselective CRH receptor antagonist, α-helical CRH, into the LS inhibit play fighting. The double labeling results indicate that the DBB may be the source area of CRH neurons involved in regulating play fighting. Compared with the specific effect of AVP in the AH on the activation of this behavior, CRH in the LS has a more general control on many aspects of play fighting, including attacks, pins, and total contact time. The effect of CRH on attacks is similar as AVP in the AH. However, the effect of CRH on pins is interesting since pins have been interpreted as a playful component in play fighting in rodents (Pellis and Pellis,

1991; Reinhart et al., 2004; Siviy et al., 2003). The current finding indicates that, compared with the specific effect of AVP in the AH on the activation of play fighting, CRH in the LS has a more general effect on regulating this behavior. CRH facilitates both playful and attack components of this agonistic behavior in juvenile hamsters. Treatment of α-helical CRH decreases the total contact time. This result also supports the idea that CRH has a more general, maybe motivational, effect on controlling play fighting compared with AVP.

The LS receives a massive input from the hippocampus and has a major output to the AH (Numan, 2000; Jakab and Leranth, 1995). This rostrocaudal connection has been consistently found in rodents, including rats and guinea pig (Jakab and Leranth, 1995; Staiger and Nurnberger, 1991a,b). The LS also receives a dorsoventrally topographic projection from the DBB (Jakab and Leranth, 1995; Staiger and Nurnberger, 1991a,b). These findings indicate a three-dimensional, rostrocaudal and dorsoventral, organization of the LS for receiving and passing information between brain areas (Staiger and Nürnberger, 1991a,b). In hamsters, the connections of the LS have only been partially described. There is a strong interconnection between the LS and the AH for controlling agonistic behaviors in hamsters (Ferris et al., 1990b). Since the hippocampus–LS-AH connection is consistently found in rodents, I predict that this connection also exists in hamsters. In addition, during an agonistic contact, the LS may receive olfactory input through the olfactory bulb to the BST and then to the LS connections reported in hamsters (Kevetter and Winans, 1981; De Vries and

Buijs, 1983). Based on these anatomical connections of the LS, it is possible that during a resident/intruder encounter, the LS integrates social memorial information from the hippocampus and olfactory information from the olfactory bulb to the BST, and generally control play fighting, or pass the information to the AH for a specific control of attacks.

The LS has been reported to be involved in regulating several kinds of social behaviors, including pair bonding, social memory, maternal behavior, and aggression in rodents (Insel et al., 1994; Dantzer et al., 1988; Slotnick and Nigrosh, 1975; Albert and Chew, 1980). CRH in the LS have been shown to play a role in stress-induced freezing or maternal defense behaviors in rats (Bakshi et al., 2002; D'Anna and Gammie, 2009). These reports partially support the idea that the LS has a general role in controlling social behaviors. It is possible that, in juvenile animals, the LS controls all agonistic behaviors, including both playful and aggressive play fighting. The AH only facilitates offensive play fighting.

Behavioral Sequence of Play Fighting

Based on the behavior and neuroanatomical findings, I propose a behavioral sequence of play fighting in two brain areas, the LS and the AH, of the play fighting neural circuitry. In a social conflict situation, an intruder can provide sensory (mainly olfactory) stimulus from the olfactory bulb and also trigger social memory from the hippocampus of the resident. Both kinds of information are intergraded in the LS. In

the LS, agonistic behavior is generally facilitated. It could be that the LS activates the motivation of a resident animal to respond to an intruder. The LS has strong reciprocal connections with the AH. When the information is passed from the LS to the AH, the control of play fighting is no longer general but is very specific on the attacks of this behavior (Fig. 5.2).

Figure 5.3 illustrates the behavioral sequence in the play fighting neural circuitry of a resident hamster in a resident/intruder scenario. An intruder stimulates a social memory input from the hippocampus. This stimulus goes into the LS, and then enters the play fighting neural circuitry through the LS. Information is intergrated in the play fighting neural circuitry, and different brain areas in the circuitry may have different roles in regulating the behavior output. These six brain areas in the network are strongly inter-connected and may cross talk in deciding the behavior output. Some brain areas may receive different aspects of inputs. For example, the MePD and the BST may receive sensory input, mainly olfactory information, and relay the information into this circuitry. The LS receives CRH inputs from the DBB. Since the DBB is anatomically closed to the nucleus accumbens, it may be responsible for the reward and pleasure effects of play fighting. This may explain why play fighting is more generally regulated in the LS. When the signals are conducted in the circuitry, calculations between brain areas keep going, and play fighting may be more specifically controlled in different brain areas in this circuitry. As the signals go to the AH, the attack component of play fighting is more specifically rmodulated. For

example, the AVP inputs form the NC and the mSON into the AH specifically facilitate the attacks of play fighting. The outputs from the play fighting neural circuitry may connect with different brain areas in the midbrain and the brain stem and control different motor patterns of the play-fighting behavior.

Function of the Play Fighting Neural Circuitry

Play fighting consists similar behavioral patterns, which could be observed in social, sex, and aggressive behaviors (Vanderschuren et al., 1997; Bekoff and Byers, 1998). It is suggested that play fighting provides a practice or a rehearsal of other social behaviors which juvenile animals may encounter in adults. My experimental results favor this idea. My data strongly suggests that a play fighting neural circuitry is activated before puberty in juvenile hamsters. This circuitry modulates social behaviors. Since the main (and probably the only) social behavior performed in juvenile hamsters is play fighting, this neural circuitry mainly controls play fighting. During puberty, this circuitry is modulated by the developments sex hormones. Under the influence of sex hormones, new brain areas may be recruited into the circuitry for regulating different kinds of social behaviors, like sex and aggression. Although a small portion of new brain areas may be added into this circuitry for regulating different social behaviors, there are conservations in the main brain areas involved in modulating all kinds of social behaviors. There is a main neural circuitry, which is established before puberty, and regulates social behaviors. Play fighting in juveniles

help activating and strengthening the neural connections between brain areas in this neural circuitry. Preventing play fighting could inhibit the development of this neural circuitry and affect the social behaviors regulated by this neural circuitry in adults. It has been reported that play deprivation causes abnormal developments of social, sexual, and aggressive behaviors (Vanderschuren et al., 1997; Bekoff and Byers, 1998). These findings support the idea that a core neural circuitry, activated before puberty, modulates all social behaviors through development.

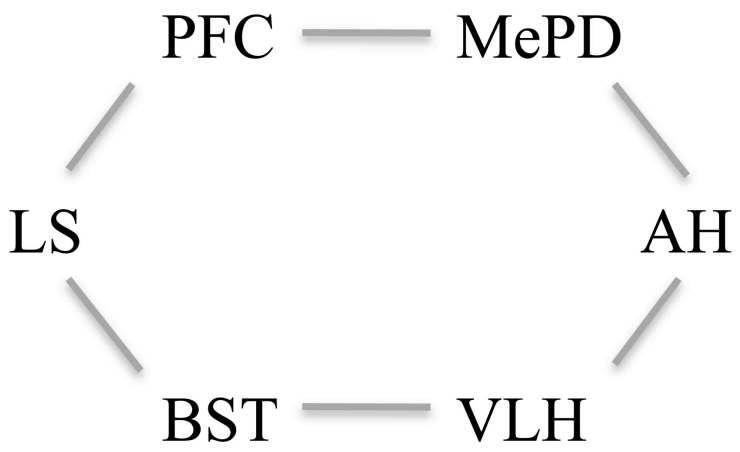
The play fighting neural circuitry reported in hamsters could possibly be applied to rat, and primates. CRH in the LS has a broad role in regulating play fighting in juvenile hamsters. Since the effect of CRH in play fighting may be motivational, it is possible that the same role of CRH in regulating play fighting exists in rats and primates as well. It is possible that the play fighting neural circuitry also exists in other rodents, and may help in regulating some aspects of complicated play behaviors in primates, including human. My research may provide a potential source in understanding the neural mechanism of play in children. The play fighting neural circuitry may also provide possible target brain areas for studying abnormal social behaviors in children, such as autism.

Conclusion

In juvenile hamsters, play fighting is controlled by a neural circuitry, including the LS, the VLH, the MePD, and the BST, centered at the AH. This behavior is

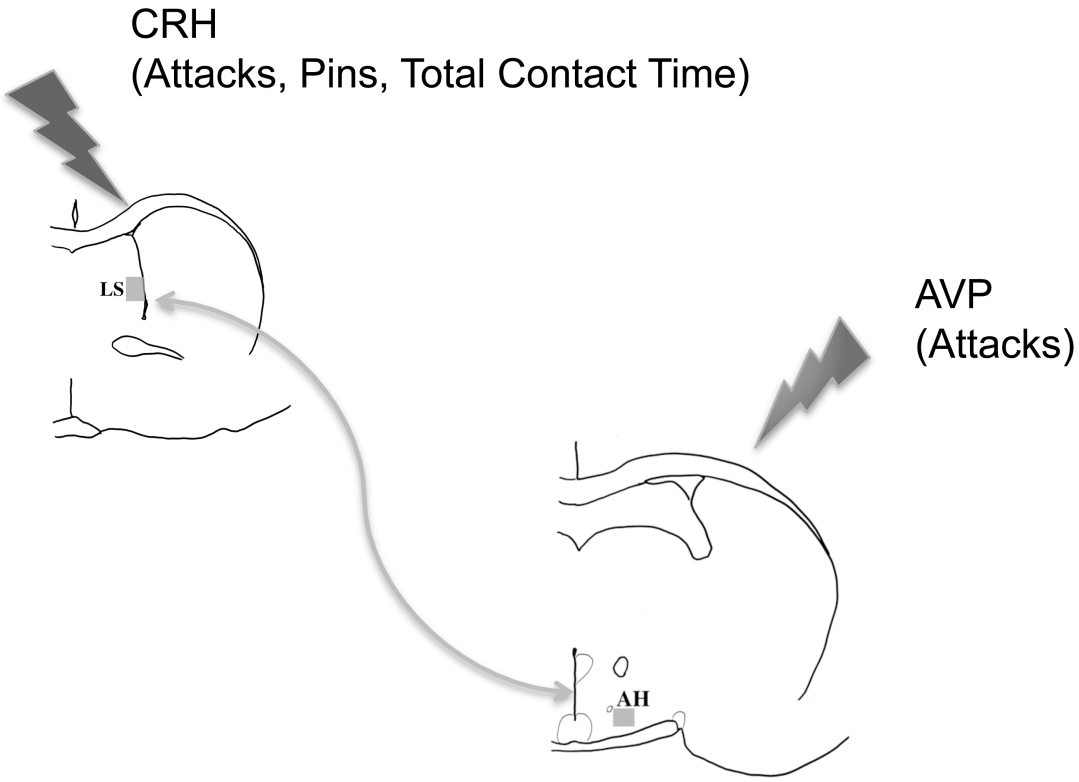
facilitated by two neurotransmitter systems, AVP and CRH. These two neurotransmitter systems work in different brain areas controlling different aspects of play fighting. CRH in the LS, possibly projecting from the DBB, provides a more general control in regulating several aspects of play fighting, including pins, attacks, and total contact time. In contrast, AVP in the AH, possibly projecting from the NC and the mSON, has a very specific role in controlling attacks of play fighting.

Figure 5.1: neural circuitry of play fighting in juvenile hamsters



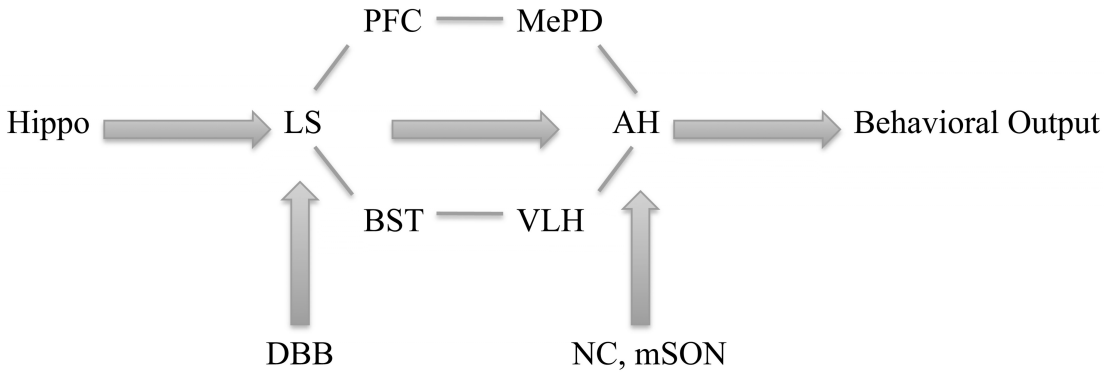
A neural circuitry of play fighting, which includes the anterior hypothalamus (AH), the lateral septum (LS), the bed nucleus of the stria terminalis (BST), the posterodorsal part of the medial amygdaloid nucleus (MePD), the ventrolateral part of the ventromedial hypothalamus (VLH), and the prefrontal cortex (PFC), modulating offensive play fighting in juvenile hamsters.

Figure 5.2: behavioral sequence of play fighting



CRH in the LS provides a more general control in regulating several aspects of play fighting, including pins, attacks, and total contact time. AVP in the AH specifically facilitates attacks of play fighting.

Figure 5.3: behavioral sequence in neural circuitry of play fighting



A behavioral sequence happened in the neural circuitry of play fighting. Several kinds of information go into the circuitry. After calculations between brain areas in this circuitry, the output from this circuitry would specifically control several aspects of the play-fighting behavior.

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